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Volume 152 Number 1

THE
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3. ***A condensed title*** or running head of no more than 35 letters and spaces should be included.

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CONTENTS

No. 1, February 1977

Blackwell, Joan F., Louis F. Gainey, Jr., and Michael J. Greenberg
Shell ultrastructure in two subspecies of the ribbed mussel, *Geukensia demissa* (Dillwyn, 1817) 1

Brehm, Paul and James G. Morin
Localization and characterization of luminescent cells in *Ophiopsila californica* and *Amphipholis squamata* (Echinodermata: Ophiuroidea) 12

Hayes, Raymond L. and Nora I. Goreau
Intracellular crystal-bearing vesicles in the epidermis of scleractinian corals, *Astrangia danae* (Agassiz) and *Porites porites* (Pallas) 26

Hedgecock, Dennis, Keith Nelson, James Simons, and Robert Shleser
Genic similarity of American and European species of the lobster *Homarus* 41

Hendler, Gordon
Development of *Amphioplus abditus* (Verrill) (Echinodermata: Ophiuroidea): I. Larval biology 51

Hori, Samuel H., Kunio Tanahashi and Norimasa Matsuoka
Morphological and cytochemical studies on the secretory granules of the pyloric caeca of the starfish, *Asterias amurensis* 64

Lang, Fred, Walter J. Costello, and C. K. Govind
Development of the dimorphic claw closer muscles of the lobster *Homarus americanus:* I. Regional distribution of muscle fiber types in adults 75

Lang, W. H. and A. M. Young
The larval development of *Clibanarius vittatus* (Bosc) (Crustacea: Decapoda; Diogenidae) reared in the laboratory 84

Moore, Carol A. and Albert F. Eble
Cytochemical aspects of *Mercenaria mercenaria* hemocytes 105

Spight, Tom M.
Availability and use of shells by intertidal hermit crabs 120

Torres, J. J., D. L. Gluck, and J. J. Childress
Activity and physiological significance of the pleopods in the respiration of *Callianassa californiensis* (Dana) (Crustacea: Thalassinidea) 134

No. 2. April, 1977

Bradshaw, William E. and Christina M. Holzapfel
Interaction between photoperiod, temperature, and chilling in dormant larvae of the tree-hole mosquito, *Toxorhynchites rutilus* Coq. 147

Conklin, Edwin J., Charles H. Bigger and Richard N. Mariscal
The formation and taxonomic status of the microbasic q-mastigophore nematocyst of sea anemones 159

Ely, Melvin J., Jr. and Arthur M. Jungreis
Radiation-induced inhibition of eclosion in the tobacco hornworm, *Manduca sexta* 169

McMahon, Robert F. and W. D. Russell-Hunter
Temperature relations of aerial and aquatic respiration in six littoral snails in relation to their vertical zonation 182

Payen, Genevieve G. and John D. Costlow
Effects of a juvenile hormone mimic on male and female gametogenesis of the mud-crab, *Rhithropanopeus harrisii* (Gould) (Brachyura: Xanthidae) 199

Potswald, Herbert E.
Further observations on the structure and function of the operculum in *Spirorbis moerchi* (Serpulidae: Spirorbinae) 209

Ram, Jeffrey L.
Hormonal control of reproduction in *Busycon:* laying of egg capsules caused by nervous system extracts 221

Schwab, Walter E.
The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita.* I. Electrophysiological analysis 233

Schwab, Walter E.
The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita.* II. The effects of ions and drugs 251

Weis, Judith S.
Limb regeneration in fiddler crabs: species differences and effects of methylmercury 263

Whitlatch, Robert B.
Seasonal changes in the community structure of the macrobenthos inhabiting the intertidal sand and mud flats of Barnstable Harbor, Massachusetts 275

Wright, Stephen H. and Grover C. Stephens
Characteristics of influx and net influx of amino acids in *Mytilus californianus* 295

No. 3, June, 1977

Brown, F. A., Jr.
Geographic orientation, time and mudsnail phototaxis 311

Carriker, Melbourne R.
Ultrastructural evidence that gastropods swallow shell rasped during hole boring 325

Conklin, Douglas E. and Luigi Provasoli
Nutritional requirements of the water flea *Moina macrocopa* 337

Fyhn, Unni E. H. and John D. Costlow
Histology and histochemistry of the ovary and oogenesis in *Balanus amphitrite* L. and *B. eburneus* Gould (Cirripedia, Crustacea) 351

Hoagland, K. Elaine
A gastropod color polymorphism: one adaptive strategy of phenotypic variation 360

Hoskin, George P. and Somsong P. Hoskin
Partial characterization of the hemolymph lipids of *Mercenaria mercenaria* (Mollusca: Bivalvia) by thin-layer chromatography and analyses of serum fatty acids during starvation 373

Lang, Fred, C. K. Govind and Joseph She
Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus:* II. Distribution of muscle fiber types in larval forms 382

Mariscal, Richard N., Edwin J. Conklin and Charles H. Bigger
The ptychocyst, a major new category of cnida used in tube construction by a cerianthid anemone 392

Ratanarat-Brockelman, Chariya
Isolation of nematode inhibitor from hemolymph of the snail, *Helix aspersa* 406

Ravindranath, M. H.
The circulating hemocyte population of the mole-crab *Emerita* (=*Hippa*) *asiatica* Milne Edwards 415

Yamaguchi, Takao
Studies on the handedness of the fiddler crab, *Uca lactea* 424

Vol. 152, No. 1 February, 1977

THE

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SHELL ULTRASTRUCTURE IN TWO SUBSPECIES OF THE RIBBED MUSSEL, *GEUKENSIA DEMISSA* (DILLWYN, 1817)[1]

Reference: *Biol. Bull.*, **152**: 1–11. (February, 1977)

JOAN F. BLACKWELL, LOUIS F. GAINEY, JR.,[2] AND MICHAEL J. GREENBERG

Department of Biological Sciences, Florida State University, Tallahassee, Florida 32306

Geukensia demissa (= *Modiolus demissus*), the ribbed mussel, is a common infaunal constituent of salt marshes on all coasts of North America. There are two subspecies. The northern one, *G. d. demissa* (Dillwyn, 1817), ranges from the Gulf of St. Lawrence to Florida (Abbott, 1974); it is also present on the west coast, having been introduced to San Francisco Bay (Hanna, 1966). The southern subspecies, *G. d. granosissima* (Sowerby, 1914), is found on both coasts of Florida and along the gulf coast to Yucatan (Andrews, 1971). The ranges overlap along the east coast of Florida, between Duval and Palm Beach Counties (unpublished collections of M. J. and P. A. Greenberg, and D. A. Price).

The two subspecies are distinguished on the basis of differences in gross shell morphology (Sowerby, 1914; Andrews, 1971; Abbott, 1974). This paper describes differences in the coloration, growth surface and ultrastructure of the shells of *G. d. demissa* and *G. d. granosissima* and suggests that these differences are characteristic of the subspecies and not of the climactic gradient coinciding with the geographical range of the species.

In 1955, Soot-Ryen assigned the common ribbed mussel, *Modiolus demissus*, to the genus *Arcuatula* on his interpretation of Jousseaume's description in Lamy (1919). Van de Poel (1959) noted that the interpretation was in error and introduced a new genus name, *Geukensia* (in honor of Professor F. Geukens of the University of Louvain). Soot-Ryen evidently concurred, for in his revision of the superfamily Mytilacea in the *Treatise on Invertebrate Paleontology* (1969), he referred the Atlantic ribbed mussel [*Modiola plicatula* Lamarck, 1819 (= *Modiola demissa* Dillwyn, 1817)] to the genus *Geukensia* Poel, 1959 as the type species, now *G. demissa* (Dillwyn, 1817).

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[1] This work was supported by U.S.P.H.S. Grant HL-09283; it is Contribution Number 29 from the Tallahassee, Sopchoppy and Gulf Coast Marine Biological Association.

[2] Present address: Department of Biological Sciences, University of Maine at Portland-Gorham, Portland, Maine 04103.

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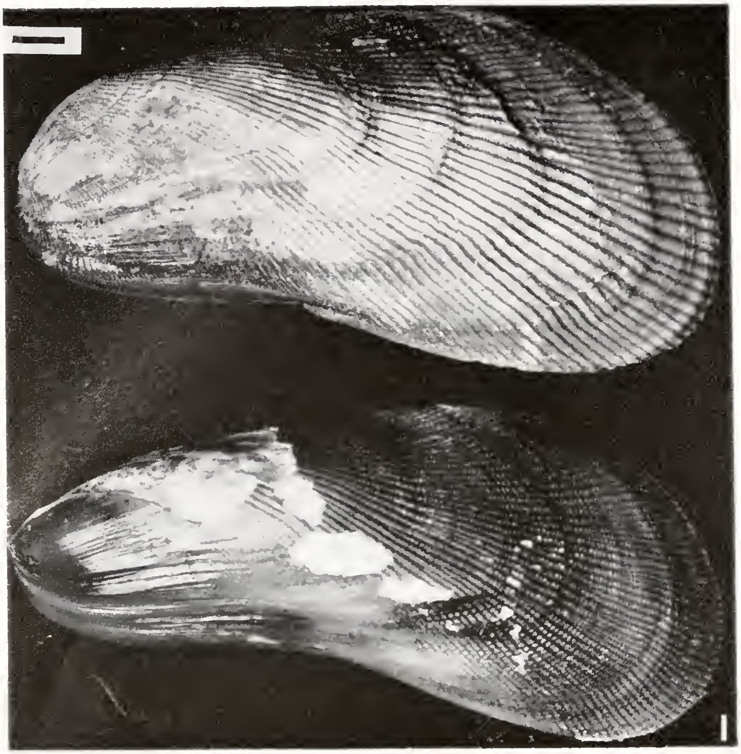


Figure 1. Shells of *Geukensia d. demissa* (top) with smooth wide ribs and *G. d. granosissima* (bottom) with narrow granulated ribs. Scale bar is 5 mm.

Recent manuals have been employing *Geukensia* as the name of the genus (*e.g.,* Andrews, 1971; Abbott, 1974). Although Keen and Coan (1974) have made *Geukensia* a subgenus of *Ischadium,* following the analysis and recommendations of Kenk (1966), the hard- and soft-part morphological and the physiological differences discussed by Pierce (1973) suggest that *Geukensia* should be a separate genus.

Materials and Methods

Specimens of *Geukensia demissa demissa,* 7 to 9 cm long, were collected from salt marshes in Oyster Creek, Maine; Oyster Bay, Long Island, New York; Sippewiset Marsh on Cape Cod, Massachusetts; Sapelo Island, Georgia; and Duval and Palm Beach Counties in Florida. Specimens of *G. d. granossima,* 6 to 8 cm long, were collected in Florida from Duval, Volusia, Franklin and Sarasota counties. The soft parts were removed and the shells retained for sectioning.

Three kinds of observations were made. To examine the growth surface, the shells were cut parallel to the ribs, rinsed in distilled water, then bleached in 5.25% sodium hypochlorite (Clorox) for 1.5 to 2 hours to loosen the periostracum. The periostracum was then gently removed with forceps. To examine the shell

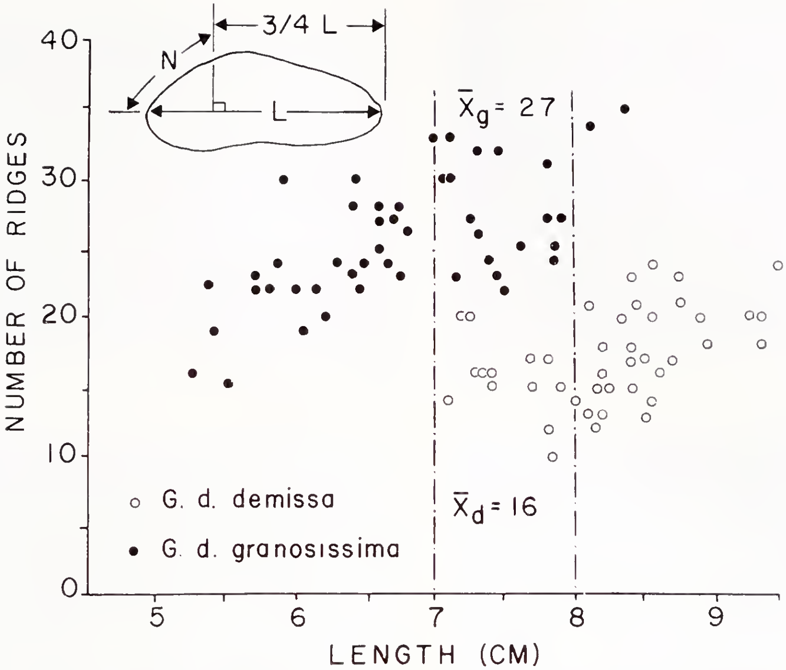


FIGURE 2. Relationship between the number of ribs and the size of the shell in the two subspecies. $\bar{x}_d$, $\bar{x}_g$ indicate the mean number of ribs in shells (*Geukensia d. demissa* and *G. d. granosissima*, respectively) between 7 and 8 cm long. The method of estimating the number of ribs (inset) was: first, at a point 3/4 of the distance from the anterior end of the shell, along the longitudinal axis (3/4 L), a line was erected perpendicular to the axis; then, the number (N) of ribs was counted along the edge subtended by the 90° angle.

layers, sections were cut parallel and perpendicular to the ribs, polished on a lap wheel, cleaned in an ultrasonic cleaner for two minutes with several washes, etched in 1% hydrochloric acid for fifteen seconds, and then bleached for 30–45 minutes. To differentiate between calcite and aragonite, etched polished sections were stained with Feigl's solution as modified by Schneidermann and Sandberg (1971). All shell sections were cut on a diamond saw.

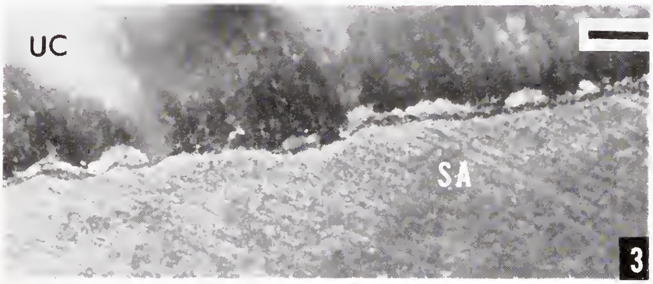
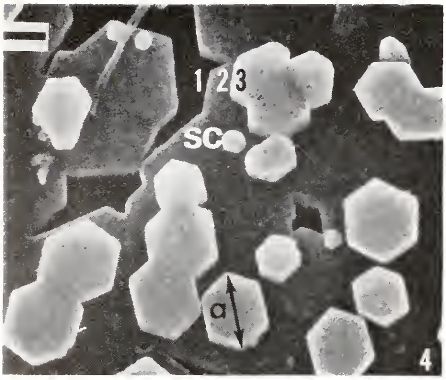
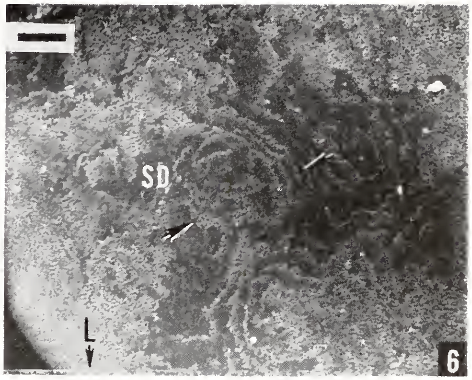
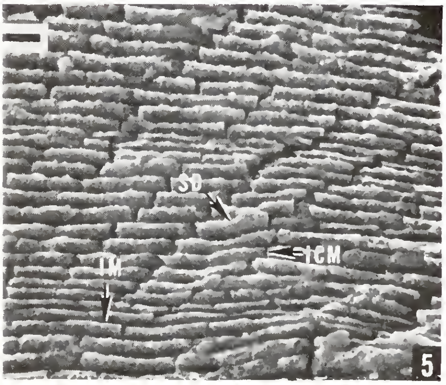
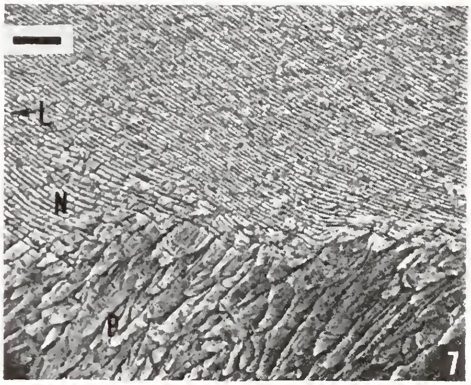


FIGURE 3. *Geukensia d. demissa:* a Feigl's stained section cut parallel to the ribs showing the outer unstained calcite (UC) and the inner stained aragonite (SA). Scale bar is 100 μm.









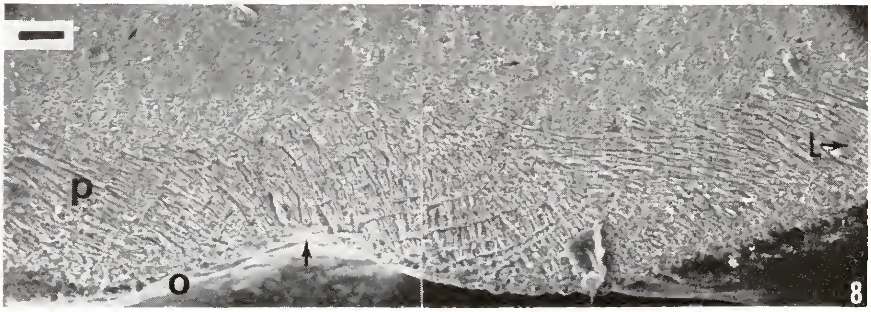


FIGURE 4. Growth surface of *Geukensia d. demissa* showing three developing layers (1, 2, 3). The hexagonal crystals are aligned with their a-axes (a) approximately in parallel. SC represents seed crystal; scale bar is 2 μm.

FIGURE 5. *Geukensia d. demissa:* polished section cut parallel to ribs showing the treppen (staircase) arrangement of the crystals. IM represents interlamellar matrix; ICM, intercrystalline matrix; SD, screw dislocation; scale bar is 2 μm.

FIGURE 6. Growth surface of *Geukensia d. demissa* showing a screw dislocation in the nacre (bracketed by arrows). L indicates direction toward lip of shell; scale bar is 43 μm.

FIGURE 7. *Geukensia d. demissa:* polished section cut parallel to ribs. The orientation of the prisms (P) to the nacre (N) is regular. Compare with Figure 8. L indicates direction toward lip of shell; scale bar is 8 μm.

Prepared sections were glued to stubs and coated with gold palladium in a vacuum evaporator (Denton DV502). The shells were examined with a scanning electron microscope (Cambridge Stereoscan Mark 2-A or S4-10).

To contrast the difference in coloration the shells were bleached eight to ten hours in 5.25% sodium hypochlorite (Clorox); this treatment removed all of the periostracum.

RESULTS

The gross shell morphologies of *G. d. demissa* and *G. d. granosissima* are easily distinguished (Sowerby, 1914; Abbott, 1974). In specimens of the same size, *G. d. granosissima* shells have numerous, narrow, granulated (or beaded) ribs; the ribs of *G. d. demissa* are fewer in number, wider and smooth (Fig. 1). The difference in rib number is immediately apparent (Fig. 1) and easily quantified (Fig. 2).

The shell of both subspecies consists of a thick calcified portion overlaid by a thin outer organic periostracum. The calcified part of the shell is divided into three layers: innermost is a nacreous layer (approximately 1 mm thick) comprising the major portion of the shell; a middle prismatic layer (0.1 mm thick) follows; and a thin (1 μm) prismatic layer is outermost. Both the nacreous and middle prismatic layers stain with Feigl's solution and are aragonite (Fig. 3). The thin outer layer does not stain and is calcite.

The nacreous layer

The nacreous layer is composed of laminae of contiguous hexagonal crystals (Fig. 4). Occasionally, rounded crystals were observed; these probably formed during periods of rapid growth (Wada, 1961). The intercrystalline space, 50–60 nm wide, contains the intercrystalline matrix in unbleached sections (Fig. 5). The interlamellar space, 100–150 nm thick, is occupied in unbleached specimens by the interlamellar matrix (Fig. 5). The crystals are elongated to varying degrees along the crystallographic a-axis and are oriented with their a-axes parallel to each other (Fig. 4) and to the inner surface of the shell. In cross section, the arrangement corresponds to the "treppen pattern" (Fig. 5) described by Schmidt (1923).

Screw dislocations were observed on the growth surface (Fig. 6) and also in polished sections (Fig. 5) on both subspecies. In Figure 4, small seed crystals are seen on, or close to, the edges of the parent crystal on the 001 face, indicating that crystal formation may be occurring by the mechanism of screw dislocation as proposed by Taylor, Kennedy and Hall (1969).

The middle prismatic layer

The middle prismatic layer in both subspecies is composed of conical aragonite prisms, the cones measuring 10 μm at their bases and 20–30 μm in height. The

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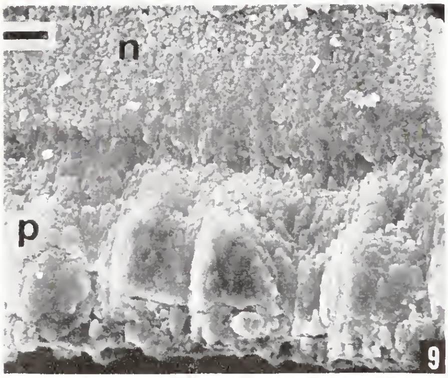
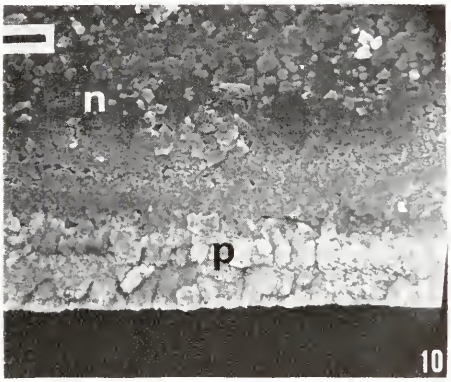
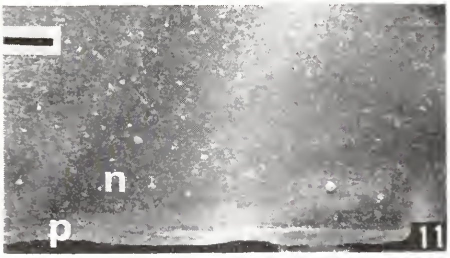
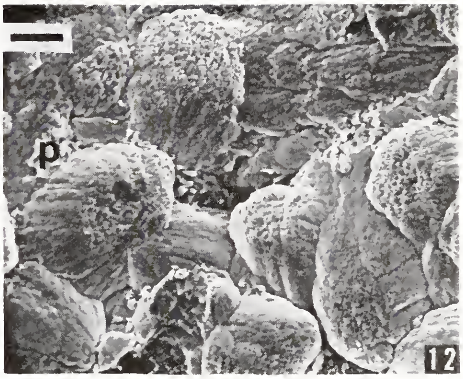
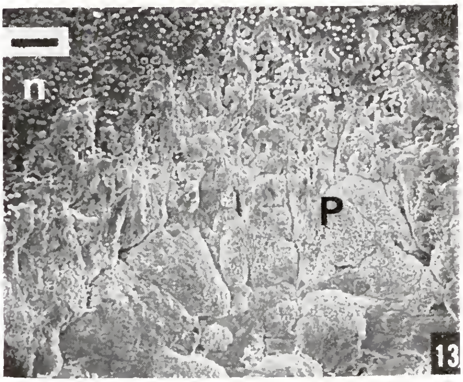
FIGURE 8. *Geukensia d. granosissima:* polished section cut parallel to the ribs. The orientation of the aragonite prisms (P) varies from 0° to 90° in a granule. The arrow indicates the anterior end of a granule. L indicates direction toward lip of shell; o represents outer surface of the shell; scale bar is 40 μm.











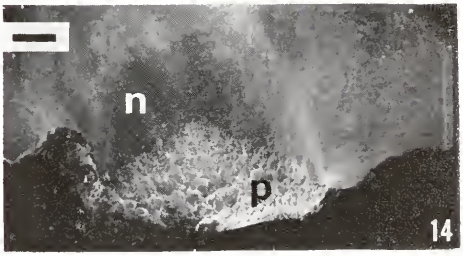


FIGURE 9. *Geukensia d. demissa:* enlargement of Figure 10 showing conical prisms in the prismatic layer (p); n represents nacre; scale bar is 10 μm.

FIGURE 10. Enlargement of Figure 11 showing regular interface between nacre (n) and prismatic (p) layers. Compare with Figure 13. Scale bar is 18 μm.

FIGURE 11. Growth surface of *Geukensia d. demissa* showing the prismatic layer (p) along the shell edge. Compare with Figure 14; n represents nacre. Scale bar is 100 μm.

FIGURE 12. *Geukensia d. granosissima:* enlargement of Figure 13 showing irregular arrangement of conical prisms in the prismatic layer (p) on the underside of a granulated rib. Scale bar is 10 μm.

FIGURE 13. *Geukensia d. granosissima:* enlargement of Figure 14. The interface between the nacreous (n) and prismatic layers (p) is curved along the posterior edge of the granule. Scale bar is 18 μm.

arrangement of the prisms is similar to the flabellate structure found in some mytilids (Oberling, 1964).

The bulk of each of the conspicuous granules or beads on the ribs of *G. d. granosissima* is composed of prismatic aragonite. In the development of each granule, the prisms are deposited in a fanned array such that the angle between the columns of prisms and the nacreous-prismatic interface varies from nearly 0° to almost 90° (Fig. 8) from one side of the granule to the other. In *G. d. demissa,* which has smooth ribs, the arrangement of aragonite cones is more regular (Figs. 7, 9, 10, 11). The difference is evident in polished sections (compare Fig. 7 and Fig. 8) and in sections of the growth surface (compare Figs. 9, 10, and 11 to Figs. 12, 13, and 14). In *G. d. demissa,* the prisms are perpendicular to the nacre in the plane of the ribs, and the angle is constant (Fig. 7).

Along the growth surface in *G. d. demissa,* the prismatic aragonite forms a layer of uniform thickness across the lip of the shell (Figs. 10, 11). In *G. d. granosissima,* however, the prismatic layer is visible only on the growth surface of the granulated ribs; between the ribs the nacre comes to the edge of the shell (Fig. 14).

The outer prismatic layer

The outer prismatic layer of calcite is very thin, and its ultrastructure could not be observed in stained, polished sections examined with the SEM. Nevertheless, in sections stained with Feigl's solution and examined under the dissecting microscope, the calcite layer was clearly revealed as the unstained outer surface of the shell (Fig. 3).

Shells of *G. d. demissa* consistently contain a dark purple pigment primarily visible on the outside of the shell and, in fact, contained in the thin outer prismatic layer. The pigment is revealed after the periostracum has been removed by bleaching. *G. d. granoissima* shells rarely contain this pigmentation; and even when it occurs, the coloration is of low intensity compared with the northern subspecies. Pigments in bivalves are thought to be waste products of metabolism incorporated into the shell (Comfort, 1951). Therefore, environmental differences should influence pigment deposition. However, both subspecies have a similar habitat and, in some areas (*e.g.,* Duval County, Florida), are conspecific. In these areas *G. d. demissa* is still more pigmented, suggesting that the difference in coloration is due to genetic rather than environmental variation.

Discussion

In general, temperate species of mytilids have shells composed of both calcite and aragonite, whereas tropical species have shells composed entirely of aragonite (Taylor *et al.,* 1969). In addition, the calcite in shells of the temperate species is confined to a thin outer prismatic layer. In *Mytilus,* Dodd (1963, 1964) has shown quantitative relationships between both the shell (beak) structure and the

FIGURE 14. Growth surface of *Geukensia d. granosissima* showing prismatic layer (p) on the underside of a granule; note that the nacreous layer (n) comes down to the edge of the shell on either side of the granule. Scale bar is 100 μm.

relative amount of aragonite on the one hand and the mean annual temperature of the habitat on the other. Nevertheless, although *G. demissa* has a geographical range from Nova Scotia to South Florida, we observed no differences in gross pattern of shell deposition; the shell mineralogy, throughout the range, appears to be similar to other temperate species. All of the shells of both subspecies in this study have a thin outer calcite layer. Bøggild (1930) found that, in *Mytilus edulis,* as in *Geukensia demissa,* the pigment is deposited in the outer layer of the shell. This may be a general characteristic of temperate mytilid shells.

In the middle prismatic layer, the subspecific difference in the deposition of the prisms is seen in the external gross morphology and on the growth surface (Figs. 9–14). In *G. d. granosissima* the granules are built up along the ribs as the prisms are deposited. The growth surface shows the underside of a fully developed granule (Fig. 14). As the next granule begins, nacre is deposited over the preceding granule. Since there are no granules in the troughs between the ribs, the nacre is laid down nearly to the edge over the prismatic layer. In *G. d. demissa,* the ribs are smooth so that the deposition of the prismatic layer occurs evenly along the lip of the shell in the troughs as well as the ribs (Fig. 11). Nacre is then laid down uniformly over the prismatic layer.

The inner nacreous layer comprises the bulk of the shell in both subspecies. The formation of new nacre crystals is not fully understood. Wada (1961, 1970) proposed several mechanisms involving screw dislocations. The position of small seed crystals developing near the edges of the 001 face of the underlying crystals (*e.g.,* Fig. 4) indicates that new crystallite formation occurs as hypothesized in Figure 15. As the parent crystal expands laterally, an inclination or thickening occurs along the edge initiating a screw dislocation and consequently a new crystal.

Growth of the nacre crystals occurs laterally along the a- and b-axes and vertically along the c-axis (axes indicated in Fig. 15). Lateral expansion of the crystals occurs concomitantly in several layers (*e.g.,* Fig. 4; Wise and Hay, 1968) and continues until a solid sheet is formed (Wada, 1968). Vertical development of the crystals along the c-axis is limited, possibly by the formation of insoluble interlamellar matrix over the crystal (proposed by Watabe, 1965; Wada, 1968). Discontinuities in the matrix could allow screw dislocations to occur (Taylor *et al.,* 1969; Bevelander and Nakahara, 1969). In fact, we have seen screw dislocations arising along the growth surface as the shell thickens (Fig. 6).

The regular orientation of nacre crystals in localized regions of the shell is characteristic of most molluscs (Wilbur, 1972). In Figure 4, for example, the crystals are aligned so that their axes are roughly parallel. Several mechanisms (reviewed by Grégoire, 1972) have been suggested to explain the uniform arrangement. Exogenous factors, such as the direction of growth of the mantle, the fibrillar arrangement in the matrix, and the direction of extrapallial fluid currents, have been proposed to explain growth and orientation of the crystals. A combination of factors is probably responsible for crystal alignment. The function of the mantle in the formation of the matrix may also include the proper orientation of the matrix fibrils seen by Mutvei (1969) and Wada (1970). If crystal nucleation occurs by screw dislocation (Fig. 15), the crystallographic axes of the seed crystal would correspond to those of the parent crystal. The whole mechanism

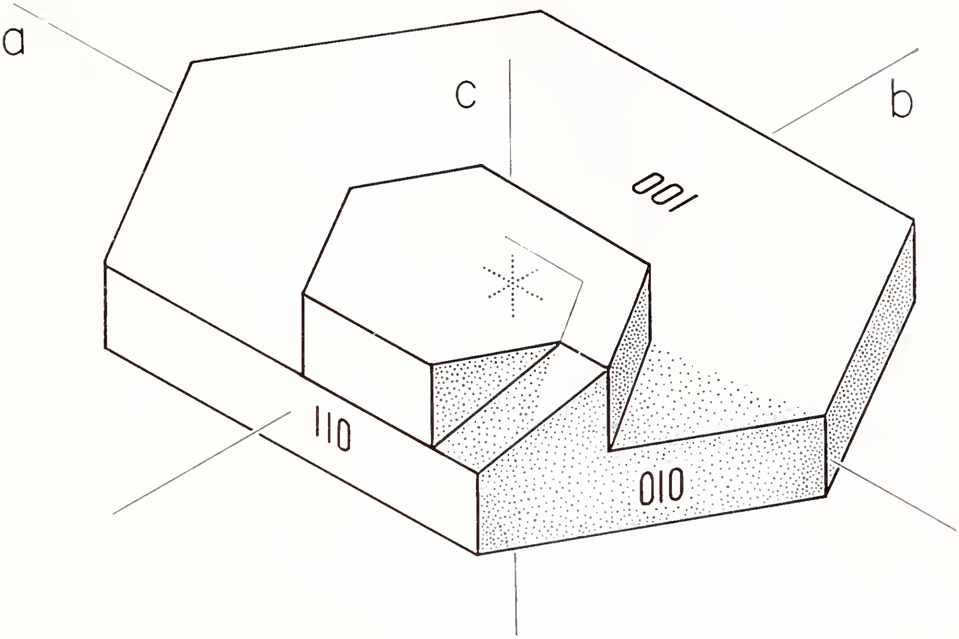


FIGURE 15. Three dimensional diagram of a screw dislocation in hexagonal nacre crystal with crystallographic axes: a, b, c; faces: 001, 110, 010. The insoluble matrix limits the growth along the c axis; discontinuities in the matrix allow the crystal to continue growing vertically and to form a new small seed crystal with axes and faces corresponding to those of the underlying crystal.

can be described as an interdependent relationship; the mantle directly influences the fibril direction in the matrix which, along with the already developed parent crystal, orients the axes of the seed crystals.

The gross differences in shell morphology seen in the two subspecies reflect the variation in deposition of the prismatic aragonite. The differences in ultrastructure cited here are a result of genetic rather than environmental influences.

The authors wish to thank Peter A. Greenberg, David A. Price and Dr. William J. Tiffany, III, for contributing specimens from their personal collections for use in this study, and also William I. Miller, III, for his assistance in the use of the scanning electron microscope.

SUMMARY

1. The shells of two subspecies of the mussel, *Geukensia demissa*, were examined with the scanning electron microscope. In both subspecies, the shells contain an inner nacreous and middle and outer prismatic layers.

2. The inner nacreous layer is composed of laminae of contiguous, hexagonal, aragonite crystals.

3. The middle layer is composed of aragonite prisms arranged in a flabellate pattern.

4. Two subspecific differences in ultrastructure, reflecting the gross morphological subspecific variation in ribbing, were evident in the middle prismatic layer. First, in *G. d. granosissima*, which has narrow granulated ribs, the orientation of the aragonite prisms with respect to the nacre changes from 0° to 90° from one side to the other of each granule; *G. d. demissa* has no granules and the prisms are always perpendicular to the nacre.

Secondly, in *G. d. demissa*, the lip of the shell is a continuous band of the prismatic layer. In *G. d. granossisima*, however, the lip is primarily nacre interspersed with clusters of aragonite prisms corresponding to the underside of a granulated rib.

5. The subspecific difference in the deposition of prismatic aragonite is genetic rather than environmental.

6. The thin outer layer could only be observed after specific staining for calcite. This layer also contains the purple pigment found commonly in *G. d. demissa* but rarely in *G. d. granosissima*.

7. A model involving screw dislocation is presented to explain the regularity of nacre crystal orientation.

LITERATURE CITED

Abbott, R. T., 1974. *American Seashells*, 2nd ed., Van Nostrand Reinhold Co., New York, 663 pp.

Andrews, J., 1971. *Sea Shells of the Texas Coast*, University of Texas Press, Austin, 298 pp.

Bevelander, G. and H. Nakahara, 1969. An electron microscope study of the formation of the nacreous layer in the shell of certain bivalve molluscs. *Calcified Tissue Res.* **3**: 84–92.

Bøggild, O. B., 1930. The shell structure of the mollusks. *Mem. Acad. Roy. Sci. Let. Danemark Sec. Sci.* (9), **2**: 232–326.

Comfort, A., 1951. The pigmentation of molluscan shells. *Biol. Rev.*, **26**: 285–301.

Dodd, J. R., 1963. Palaeoecological implications of shell mineralogy in two pelecypod species. *J. Geol.*, **71**: 1–11.

Dodd, J. R., 1964. Environmentally controlled variation in the shell structure of a pelecypod species. *J. Paleontol*, **38**: 1065–1071.

Grégoire, C., 1972. Structure of the molluscan shell. Pages 45–102 in M. Florkin and B. Scheer, Eds., *Chemical zoology, Volume VII: Mollusca*. Academic Press, New York.

Hanna, G. D., 1966. Introduced mollusks of western North America. *Occas. Pap. Calif. Acad. Sci.*, **48**: 1–108.

Keen, A. M. and E. Coan, 1974. *Marine molluscan genera of western North America*, Stanford University Press, California, 174 pp.

Kenk, V. C., 1966. A revision of Brachidontes (Mollusca-Bivalvia). *Ph.D. thesis, Harvard University*, Cambridge, Massachusetts.

Lamy, E., 1919. Les moules et les modioles de la Mer Rouge (D'après les materiaux recueillis par M. le Dr. Jousseaume). *Bull. Mus. Nat. Hist. Natur. Paris*, **25**: 173–178.

Mutvei, H., 1969. On the micro- and ultrastructure of the conchiolin in the nacreous layer of some recent and fossil molluscs. *Stockholm Contrib. Geol.*, **20**: 1–17.

Oberling, J. J., 1964. Observations on some structural features of the pelecypod shell. *Mitt. Naturforsch. Ges. Bern*, **20**: 1–63.

Pierce, S. K., Jr., 1973. The rectum of "*Modiolus*" *demissus* (Dillwyn) (Bivalvia: Mytilidae): a clue to solving a troubled taxonomy. *Malacologia*, **12**: 283–293.

Schmidt, W. J., 1923. Bau und bildung der Perlmuttermasse. *Zool. Jahr. Abt. Anat. Ontog. Tiere*, **45**: 1–148.

Schneidermann, N. and P. A. Sandberg, 1971. Calcite-argonite differentiation by selective staining and scanning electron microscopy. *Trans. Gulf Coast Ass. Geol. Soc.*, **21**: 349–352.

Soot-Ryen, T., 1955. A report on the family Mytilidae. *Allen Hancock Pacific Expeditions*, **20**: 1–174.

Soot-Ryen, T., 1969. The superfamily Mytilacea. Pages 271–280 in R. C. Moore, Ed., *Treatise on invertebrate paleontology*, University of Kansas, Kansas.

Sowerby, G. B., 1914. Description of new species of Mollusca from New Caladonia, Japan and other localities. *Proc. Malacol. Soc. London*, **11**: 5–10.

Taylor, J. D., W. J. Kennedy and A. Hall, 1969. The shell structure and mineralogy of the Bivalvia. Introduction. Nuculacea-Trigonacea. *Bull. Brit. Mus. (Natur. Hist.) Zool. Suppl.*, **3**: 1–125.

Van de Poel, L., 1959. Faune malacologique de Hervien, Troisieme note (première partie). *Bull. Inst. Roy. Sci. Natur. Belg.*, **35**: 1–26.

Wada, K., 1961. Crystal growth of molluscan shells. *Bull. Nat. Pearl Res. Lab. Kashikojima*, **7**: 703–828.

Wada, K., 1968. Mechanism of growth of nacre in Bivalvia. *Bull. Nat. Pearl Res. Lab. Kashikojima*, 13: 1561–1586.

Wada, K., 1970. Studies on the calcified tissue in molluscs XVII. Pages 226–244 in H. Yukawa, Ed., *Profiles of Japanese science and scientists*, Kodansha Ltd., Tokyo.

Watabe, N., 1965. Studies of shell formation VIII. Crystal-matrix relationships in the inner layers of mollusc shell. *J. Cell Biol.*, **18**: 701–703.

Wilbur, K. M., 1972. Shell formation in mollusks. Pages 103–154 in M. Florkin and B. Scheer, Eds., *Chemical zoology, Volume VII: Mollusca*. Academic Press, New York.

Wise, S. W., Jr. and W. W. Hay, 1968. Scanning electron microscopy of molluscan shell ultrastructures. II. Observations of growth surfaces. *Trans. Amer. Microsc. Soc.*, **87**: 419–430.

Reference: *Biol. Bull.*, **152**: 12–25. (February, 1977)

LOCALIZATION AND CHARACTERIZATION OF LUMINESCENT CELLS IN *OPHIOPSILA CALIFORNICA* AND *AMPHIPHOLIS SQUAMATA* (ECHINODERMATA: OPHIUROIDEA)

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The identification of light-emitting cells is central to the understanding of the control of bioluminescence. In many phyla little is known of the morphology and organization of the luminescent cells, primarily because techniques have not been available for spatially detecting low light levels. Within the echinoderms, luminescent representatives of the class Ophiuroidea have long been recognized (Harvey, 1952). However, luminescence has recently been observed in certain Holothuroidea, Crinoidea, and Asteroidea, as well as confirmed in the Ophiuroidea (Herring, 1974).

In the first decade of this century, a concentrated but inconclusive effort was made to locate the photogenic cells in ophiuroids (Sterzinger, 1907; Reichensperger, 1908a; Trojan, 1909a; Sokolow, 1909). The results of most of these investigations led to the opinion that some type of epidermal gland cell was involved, but different cells were implicated by various authors. Very little information appeared subsequently until Buchanan (1963) reported further details of the luminescent cells first described by Reichensperger (1908a) in *Amphipholis* (= *Amphiura*) *squamata* and *Amphiura filiformis*. He described this cell type as being comprised of a pyriform, nucleated cell body deep within the spine. The cell body tapered to an elongate duct which could reach long distances to the surface cuticle. The resemblance of these large cells, termed photocytes, to neighboring mucous glands suggested that the cells were of glandular origin (Buchanan, 1963). Extracellular luminescence was suggested by early observers (Reichensperger, 1908b; Trojan, 1909a), who indicated that the cells communicated with the exterior through the duct. However, there was no physiological evidence to support the theory of extracellular luminescence. Reichensperger (1908b) and Sokolow (1909) recognized that this cell did not perfectly match the distribution of observed luminescent sites. Herring (1974) also reported an inability to find any epidermal gland cell which corresponded to the areas he found to be luminescent in ophiuroids.

The purpose of the present paper is to describe: the general distribution of luminescent areas; the specific cellular sources of luminescence; and the luminescence emission spectra of *Ophiopsila californica* Clark, 1921 and *Amphipholis squamata* (Delle Chiaje, 1828). A preliminary report of this work has been published (Brehm, Morin, and Reynolds, 1973).

MATERIALS AND METHODS

Two species of ophiuroids were used in this study, *Ophiopsila californica* and *Amphipholis* (= *Axiognathus,* see Thomas, 1966 and Clark, 1970) *squamata.*

Both species were collected near the Santa Catalina Marine Biological Laboratory, California, using SCUBA at night from depths of 10 to 30 meters. *Amphipholis squamata* was also collected in shallow water at Palos Verdes Point, California, and at Odiorne Point, New Hampshire. *Ophiopsila californica* is large, and each arm can reach a length of 20 centimeters from disc to arm tip. *Amphipholis squamata* is small, and the arms only reach a length of approximately 2 centimeters. The animals were maintained at U.C.L.A. in a closed circulating seawater system at 12° C.

The distribution of luminescence in *O. californica* was studied with an image intensifier coupled to a video tape deck and viewed on a television monitor. The detailed methodology of this technique has been described (Reynolds, 1972). A second method for identifying luminescent areas involved isolating and testing specific arm parts for luminescence. The parts were chemically excited with 0.54 M KCl (isotonic to sea water) and light production was monitored using standard photometric techniques.

The luminescent sites become fluorescent after stimulation. This fluorescence was used to study the distribution of luminescence. The advantage of observing fluorescence is two-fold. First, the luminescence lasts only milliseconds and is therefore difficult to observe. The fluorescence, on the other hand, can be observed easily, because it lasts for as long as the stimulating light is maintained. Secondly, fluorescence is retained even after the tissue has been appropriately fixed and sectioned. Therefore, it is possible to see cellular detail. To prepare histological sections for fluorescence microscopy, the tissue was excited to luminesce in 0.54 M KCl. It was important that the tissue was not anesthetized (in 0.36 M $MgCl_2$ isotonic to sea water or Ca-free sea water) before the KCl treatment, since anesthetized tissue did not fluoresce.

After 12 hours in 5% formalin the arms were placed in 50 mM EDTA for approximately 72 hours until decalcification of the spines and plates was complete. The arms were dehydrated in ethanol, cleared in toluene, embedded in Tissuemat (56–58° C), and cross sections were cut between 8–20 μm thickness. These sections were stained with Harris hematoxylin and eosin, toluidine blue, thionin, or Holme's silver stain and mounted in Permount. Through each stage of this procedure, most of the natural fluorescence of the photogenic material was preserved. Fluorescence was observed through a 530 nm barrier filter following excitation from a xenon source with a Leitz BG-12 (blue) filter.

The luminescent emission spectra of *O. californica* and *A. squamata* were measured with an image intensifier coupled to a spectrophotometer and analyzed both by computer (Gruner, 1973) and a Joyce Loebl microdensitometer.

RESULTS

Distribution of the photocytes

Ophiopsila californica. A previous abstract on *Ophiopsila californica* (Brehm *et al.*, 1973) reported that image intensification showed the localization of luminescent sites. The following parts of each arm segment were demonstrated to be luminescent: spines, lateral plates, ventral plate and tentacular scales (Fig. 1). The aboral radial shields and oral plates were reported to be the only luminescent

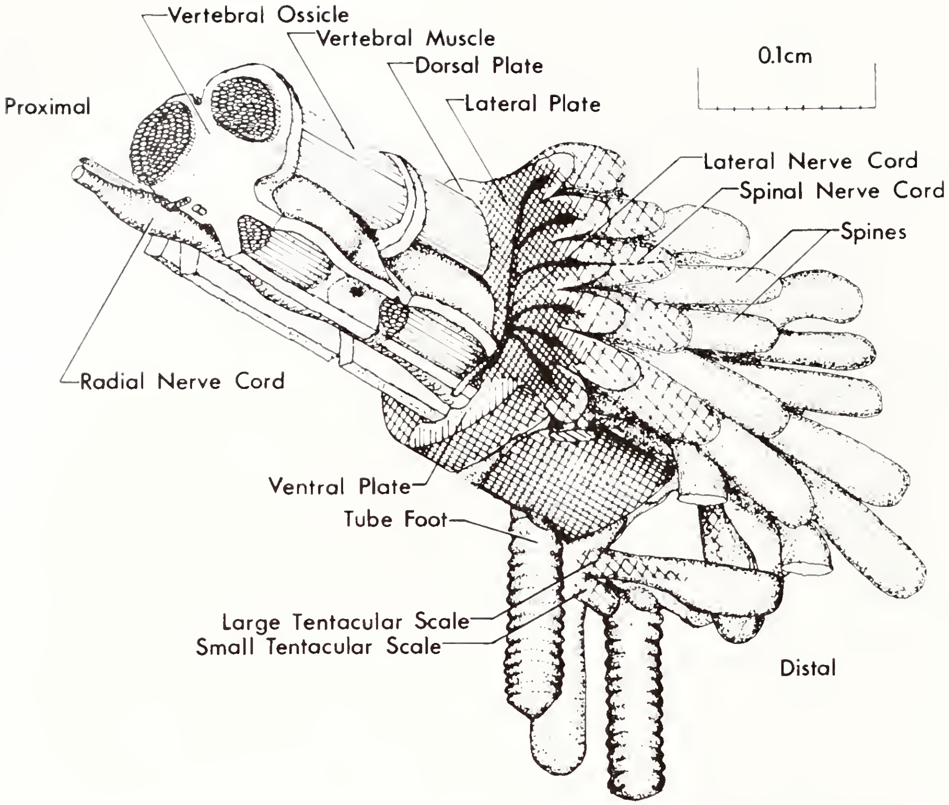
Vertebral Ossicle
Vertebral Muscle
Dorsal Plate
Lateral Plate
0.1cm
Proximal
Lateral Nerve Cord
Spinal Nerve Cord
Spines
Radial Nerve Cord
Ventral Plate
Tube Foot
Large Tentacular Scale
Small Tentacular Scale
Distal

FIGURE 1. Reconstruction of five segments of an *Ophiopsila californica* arm. Luminescent areas are represented by the cross-hatching, with closer cross-hatching indicating a greater concentration of photocytes. See text for description.

components of the disc. Subsequently, these observations were verified by photometrically testing isolated parts of each segment upon addition of 0.54 M KCl. Luminescence in the whole animal was elicited by either electrical, chemical, or mechanical stimulation, and in all cases the sites of light production were identical. There was no indication of extracellular luminescence, even with chemical stimulation by application of KCl or distilled water. All of the light appeared to be emitted intracellularly from photocytes within the trabecular skeleton.

Fluorescence microscopy was also used to identify the distribution of the luminescence. Fluorescence could be observed in both *O. californica* and *A. squamata* only after the animals were stimulated to luminesce (Figs. 2 and 4). Generally, mechanical or electrical stimulation led to only very limited fluorescence, even when accompanied by intense luminescence. KCl, isotonic to sea water, was found to produce intense luminescence as well as fluorescence. Simultaneous microscopic observation and delivery of KCl allowed a direct comparison of luminescent and fluorescent areas. The fluorescent distribution corresponds precisely to the

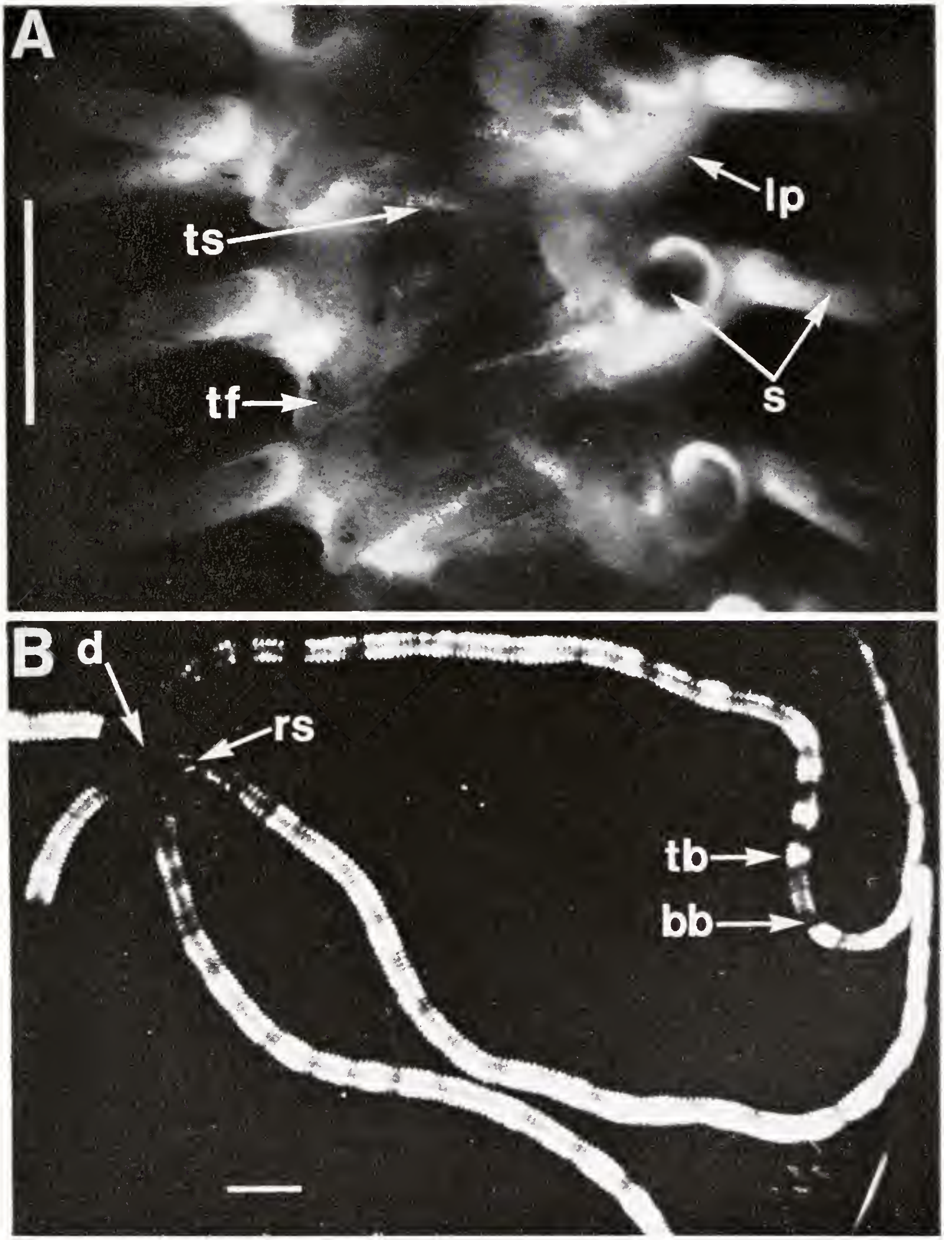

FIGURE 2. A. A ventral view of the fluorescence within three segments of an *Ophiopsila californica* arm. The arm was pretreated with 0.54 M KCl. The tube feet (tf), spines (s), tentacular scales (ts), and proximal end of the lateral plate (lp) are shown. The bar represents 1 mm. B. A luminescing *Ophiopsila californica* which illustrates the banding pattern of luminescence along the arm. Brown bands (bb) and tan bands (tb) can be discerned. Except for one pair of radial shields (rs), the disc is dark. The bar represents 1 cm. The photograph was taken with Tri-X film with an exposure time of one second.

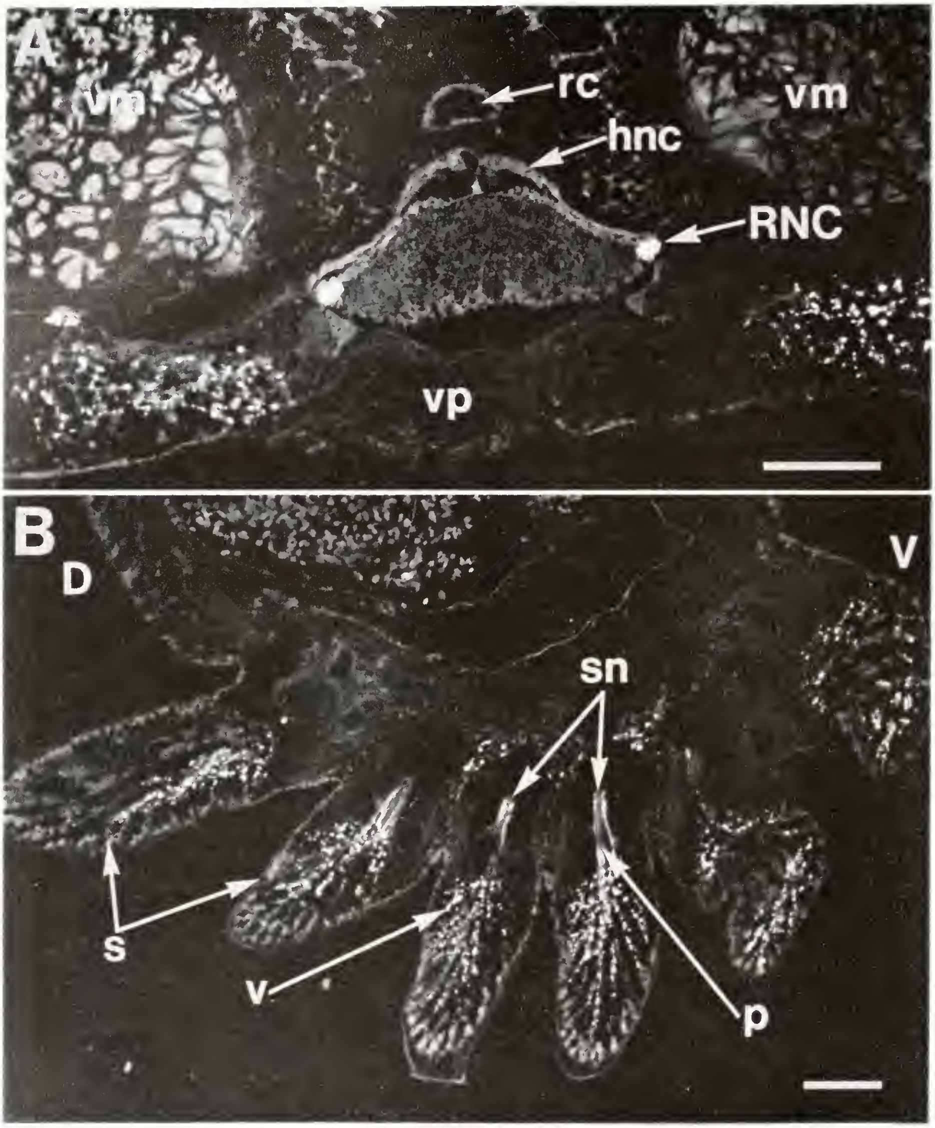

FIGURE 3. A. A 10 μm thick cross-section of the ventral region of an unstained *Ophiopsila californica* arm. The lateral sides of the ventral plate (vp) and the lateral edges of the radial nerve cord (RNC) display fluorescence. The nonluminescent hyponeural nerve cord (hnc), vertebral muscles (vm), and radial water canal (rc) are also shown. The bar represents 100 μm. B. A 10 μm thick cross-section of *Ophiopsila californica*, unstained, at the level of the spines (s). Fluorescent varicosities (v) are seen in the spines and the processes (p) are also seen within the spinal nerves (sn). The bar represents 100 μm.

distribution of luminescent areas observed by visual means and image intensification. Furthermore, the fluorescence intensity of an area was directly related to the *in vivo* capability of that area to produce light. The long term stability of the fluorescence in *O. californica* allowed a more precise identification of the luminescent areas than could be accomplished using the luminescence itself.

Figure 1 illustrates the location of the luminescent sites within several segments of an *O. californica* arm. All of the lateral spines along the arm are luminescent. However, the greatest intensity of luminescence, as judged by fluorescence, is in the basal and ventral region of each spine (Fig. 2a). Much of the lateral plate is luminescent, but the ventral portion of each plate is more luminescent than the dorsal portion. The proximal end of the lateral plate tends to be brightly luminescent, whereas the distal end is only weakly luminescent (Fig. 2a). In the distal region of the arm, the lateral plates tend to wrap around the nonluminescent dorsal plate and meet, thus resulting in a dorsal luminescence that is not present in the proximal arm. Also, all regions of the ventral plate are luminescent. Both pairs of tentacular scales are luminescent. The large scales exhibit a broad luminescence basally, which tapers to a narrow luminescent central core extending approximately half the length of the scale. The smaller tentacular scales are uniformly luminescent. Elements within the lateral region of the radial nerve cord are also luminescent; however, they are restricted to the ganglionic regions of the nerve cord (Fig. 3a). Both the lateral nerves and spinal nerves have luminescent tissue associated with them (Fig. 3b).

As noted in the preliminary paper (Brehm *et al.*, 1973), image intensification studies indicated that the intensity of luminescence in *O. californica* is related to the pigmentation pattern along the arm (Fig. 2b). Brown and tan bands alternate along the dorsal part of the arm, and each is separated by a narrow white band. The two darker bands include several arm segments, while the white bands generally include a single segment. The white band emits the most intense luminescence in the intact animal. This segment, when examined after KCl treatment, is the most intensely fluorescent region. The brown band has the weakest luminescence and fluorescence, while the intensity of the tan band is intermediate to the brown and white bands. Furthermore, it has been observed that the threshold of electrical excitability is related to the pigmentation of the segment. When the animal luminesces, tan bands can be observed to luminesce all along the arm length while brown bands often remain totally unlit (Fig. 2b).

Amphipholis squamata. This species differs from *Ophiopsila californica* in the distribution and stability of the fluorescence. Visual comparisons demonstrated that, as in *O. californica*, fluorescence is equivalent to luminescence. In *A. squamata* the luminescence and fluorescence is restricted primarily to the lateral plates (Fig. 4). Both the dorsal and ventral plates show a small amount of fluorescence, indicating that they are only weakly luminescent (Fig. 4). Similarly, the oral plates occasionally show a small amount of fluorescence. The spines are not luminescent or fluorescent. A high degree of variability exists between *A. squamata* individuals with respect to fluorescent distribution. In the New Hampshire individuals, luminescence was substantially weaker and fluorescence more sparsely distributed than in California individuals. In both populations sampled, and unlike *Ophiopsila*, the fluorescence was very labile under xenon, mercury, and even tungsten light. Blue light from a xenon source applied to the arms for three to five minutes generally produced an irreversible disappearance of all fluorescence.

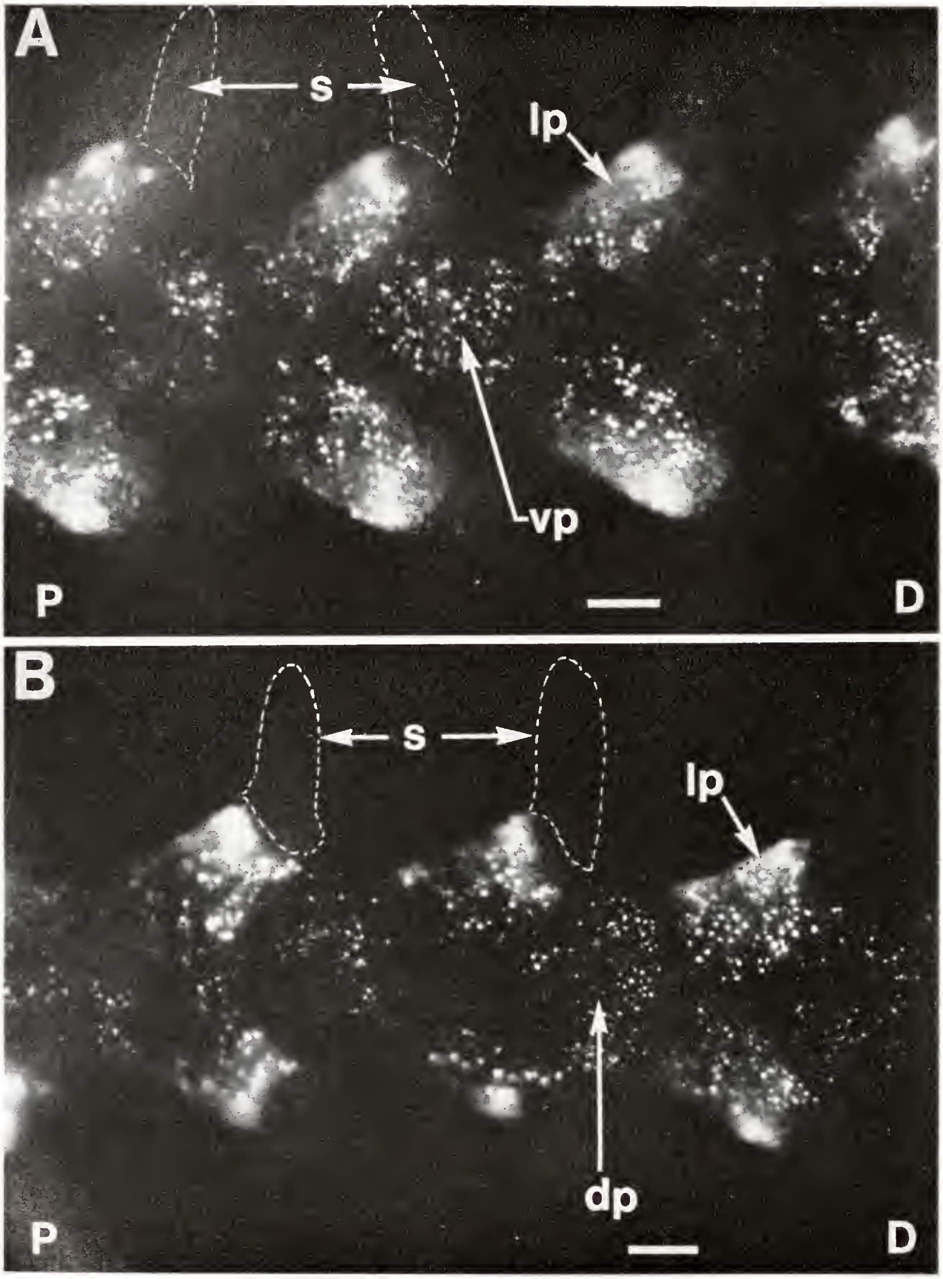

FIGURE 4. A. A ventral view of the fluorescence within four segments of an *Amphipholis squamata* arm. The arm was pretreated with 0.54 M KCl. The lateral plates (lp), ventral plate (vp), and spines (s) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are also indicated. B. A dorsal view of the same four segments of *Amphipholis squamata.* The lateral plates (lp), spines (s), and dorsal plate (dp) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are shown. The bar represents 100 μm in A and B.

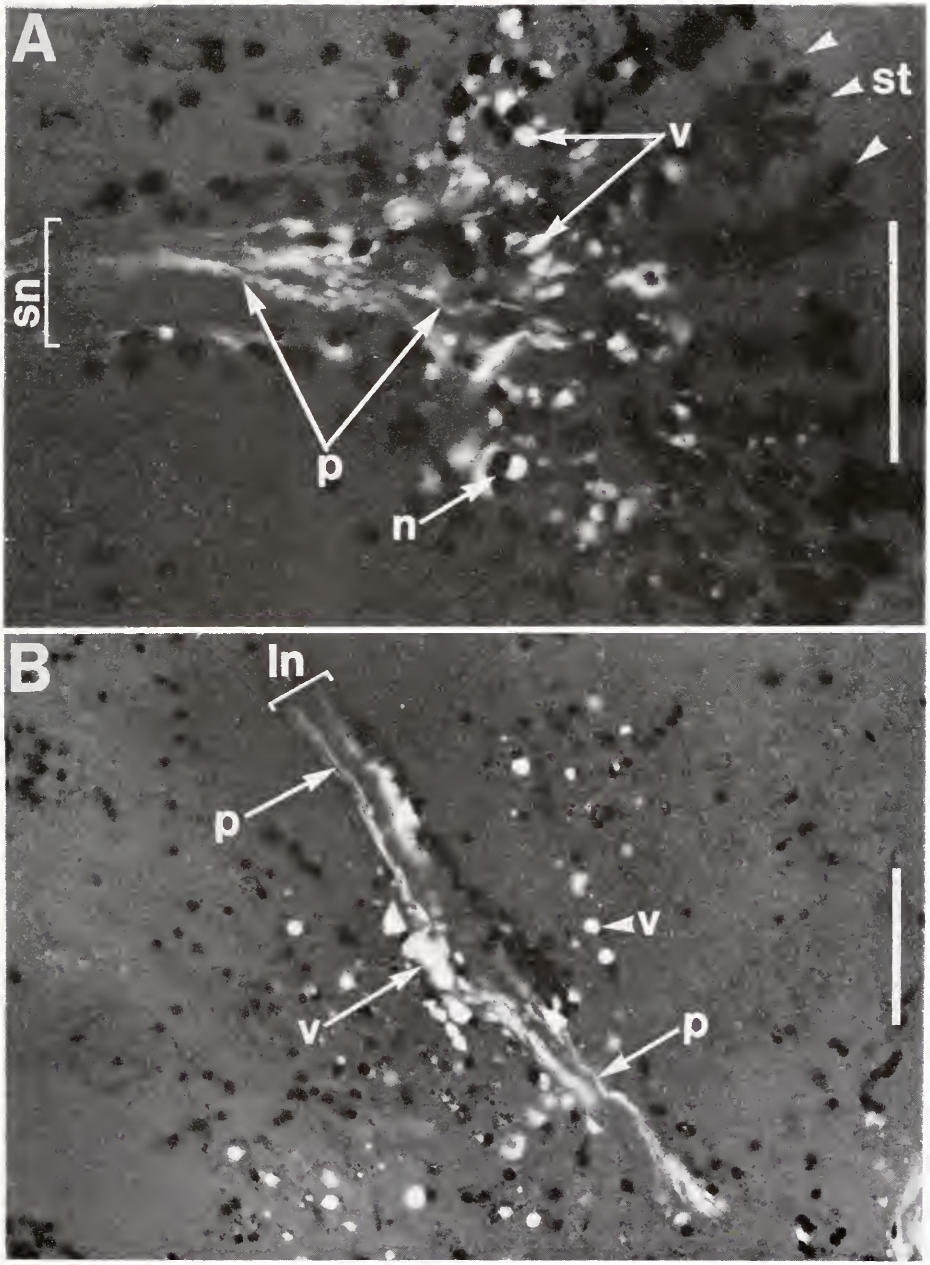

FIGURE 5. A. A longitudinal 10 μm thick section of *Ophiopsila californica* spine stained with Harris hematoxylin. Fluorescence is seen in the varicosities (v) as well as in the processes (p). The nuclei (n) of epithelial cells stain dark. The spinal nerve (sn) containing luminescent processes branches within the spine and stops short of the spine tip (st with arrows). The bar represents 100 μm. B. A longitudinal 10 μm thick section of *Ophiopsila californica* lateral nerve cord (ln) stained with Harris hematoxylin. Luminescent processes (p) are seen within the lateral nerve cord and varicosities (v) are within the surrounding tissue. The bar represents 100 μm.

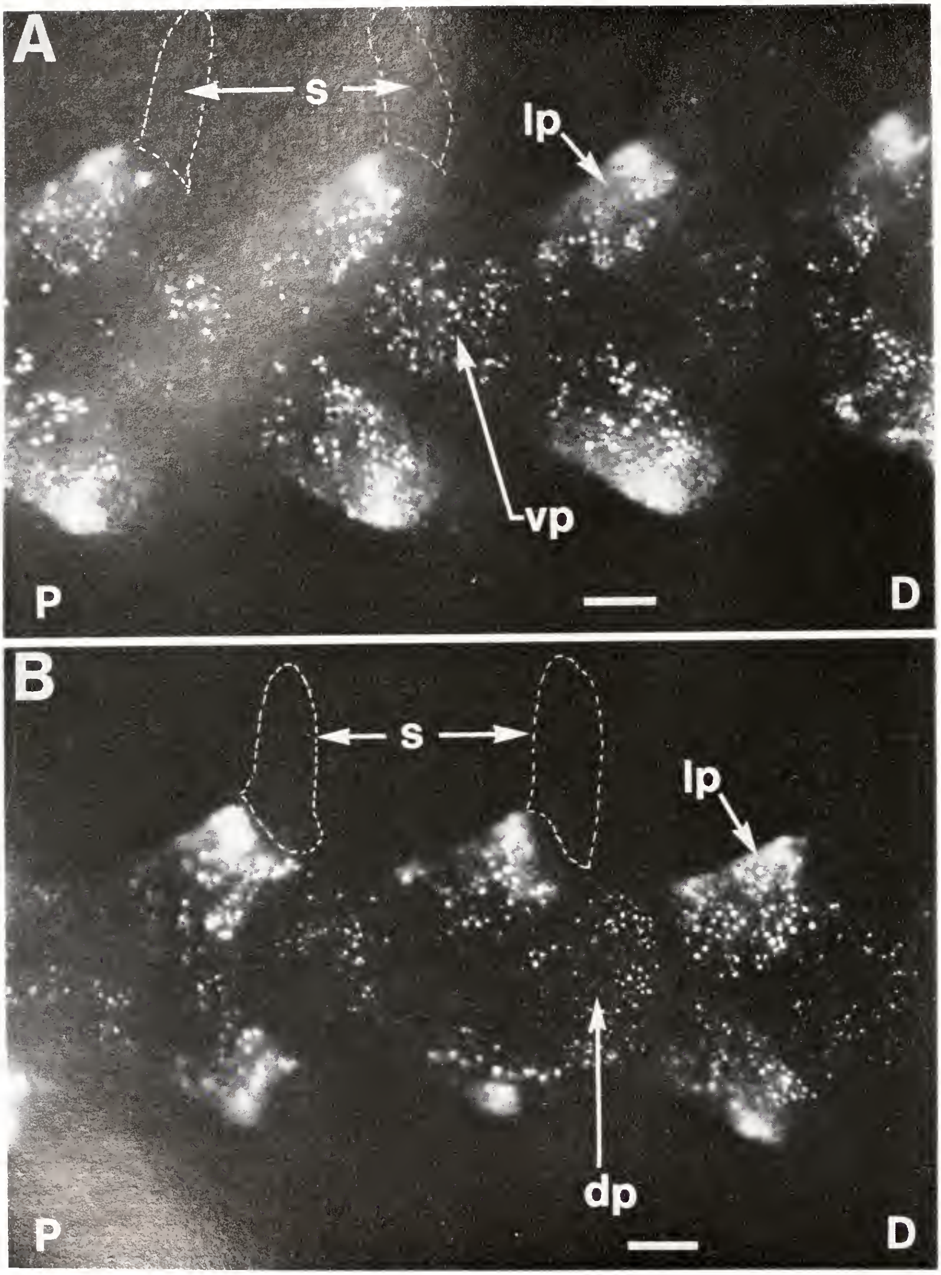

FIGURE 4. A. A ventral view of the fluorescence within four segments of an *Amphipholis squamata* arm. The arm was pretreated with 0.54 M KCl. The lateral plates (lp), ventral plate (vp), and spines (s) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are also indicated. B. A dorsal view of the same four segments of *Amphipholis squamata*. The lateral plates (lp), spines (s), and dorsal plate (dp) are labelled. The nonfluorescent spines are outlined in two segments. The proximal (P) and distal (D) segments are shown. The bar represents 100 μm in A and B.

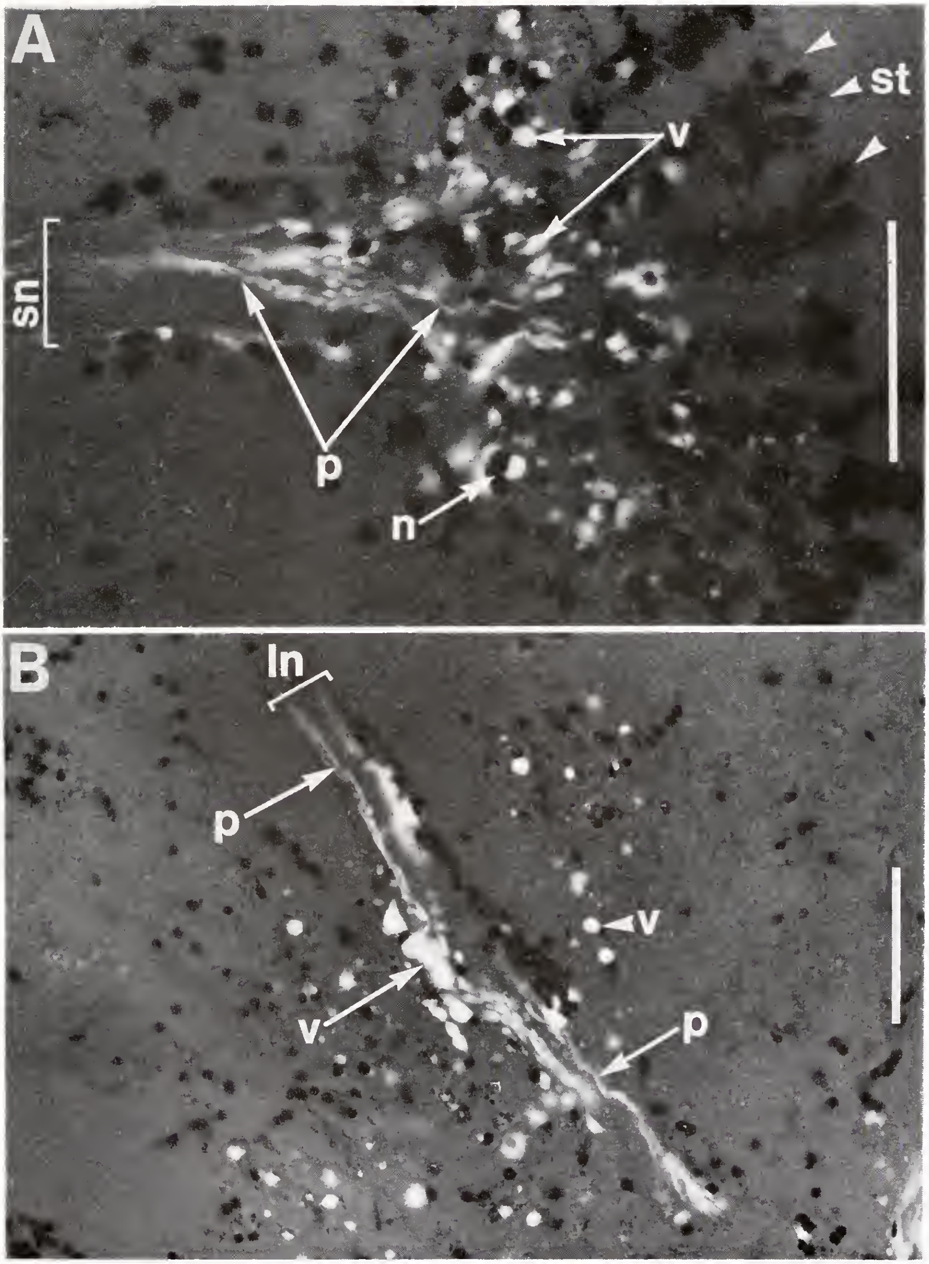

FIGURE 5. A. A longitudinal 10 μm thick section of *Ophiopsila californica* spine stained with Harris hematoxylin. Fluorescence is seen in the varicosities (v) as well as in the processes (p). The nuclei (n) of epithelial cells stain dark. The spinal nerve (sn) containing luminescent processes branches within the spine and stops short of the spine tip (st with arrows). The bar represents 100 μm. B. A longitudinal 10 μm thick section of *Ophiopsila californica* lateral nerve cord (ln) stained with Harris hematoxylin. Luminescent processes (p) are seen within the lateral nerve cord and varicosities (v) are within the surrounding tissue. The bar represents 100 μm.

Morphology of the photocytes

Fluorescence, associated with luminescent tissue, was maintained through fixation, decalcification, dehydration, and staining and could be detected in cross sections of *O. californica* (Figs. 3 and 5). Decalcification eliminated the light scattering effect of the skeleton and allowed a precise identification of the photocytes. The photocytes appear to be composed of long narrow processes which have occasional swellings, termed varicosities. Thus, the photocytes have two morphological components: varicosities and associated processes (Fig. 5). Both tend to be deep in the skeleton, but are also found in association with the nervous system. The relationship between varicosities and processes is not clear. Moreover, the boundaries of a single photocyte cannot be determined, so it is not known how many varicosities and processes constitute a single cell.

The varicosities are usually spherical and are frequently drawn out to meet an associated process, the second morphological component (Fig. 5). The varicosities average 3.0 μm in diameter with a range of 0.4 to 16 μm. Below 0.4 μm the varicosities become indistinguishable from the processes. There are thousands of these varicosities within one segment of the arm. A small percentage of the varicosities are nucleated, as judged from hematoxylin staining, and tend to be the largest within the size range. The varicosities lie amongst the cellular matrix which ramifies through the skeletal material (Fig. 5). The highest density of the varicosities is in the region immediately surrounding the ganglion at the base of each spine. The largest varicosities are found in this same area.

The processes have a diameter of less than 0.4 μm and appear to have their origin in the lateral edges of the radial nerve cord (Fig. 3a). It has been observed through electron microscopy (personal observation) that the hyponeural nerve cord wraps around the lateral edge of the radial nerve cord. Therefore, some of the observed fluorescence may also be associated with this region of hyponeural tissue. The processes are restricted to the region of radial nerve cord which is proximal to the origin of the lateral nerve cord (Fig. 1). In each segment at the end of the radial nerve cord ganglia, the processes appear to change direction and run within the lateral nerve cord (Figs. 3a and 5b). Except for their bright fluorescence, the processes are indistinguishable from the accompanying nervous tissue at the light microscopic level. The processes extend from the lateral nerve cord into the nerve bundle of the spines and can be followed as they branch off to adjoin varicosities within the spines (Figs. 3b and 5a). Processes are not always associated with nerve bundles. They are seen to connect adjacent varicosities in every luminescent area.

Varicosities and their processes, henceforth referred to as photocytes, are also present in the ventral plate (Fig. 3a), lateral plate (Fig. 5b), and tentacular scales (Fig. 2a). The absence of photocytes in nonluminescent regions such as the vertebral ossicle, dorsal plate, musculature, and the tube feet is conspicuous. The photocytes do not stain with Harris hematoxylin, toluidine blue, or thionin and are therefore quite transparent to transmitted tungsten light. They do, however, show a slight affinity for Holme's silver stain. The photocytes have never been observed to reach the surface cuticle.

Examination of the fresh tissue of *Amphipholis squamata* indicates that a similar network of varicosities and processes is responsible for the luminescence in

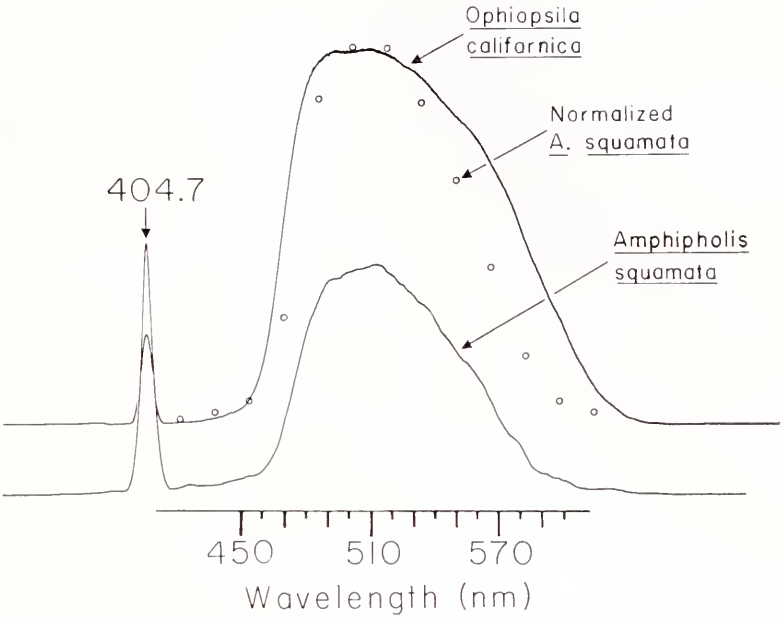

FIGURE 6. The luminescent emission spectra of *Ophiopsila californica*, *Amphipholis squamata*, and *Amphipholis squamata* normalized to the same peak amplitude as *Ophiopsila californica* (open circles). The ordinate indicates relative intensity in arbitrary units and the 404.7 nm peak is a calibration mark.

this species (Fig. 4). Since the fluorescence is less stable in this species than *O. californica*, similar histological studies were not attempted.

Emission spectra

The luminescent emission spectra of *Ophiopsila californica* and *Amphipholis squamata* (Fig. 6) are extremely broad with a similar approximate maximum at 510 nm and a half band-width of 71 nm. The emission spectra did not differ with chemical, mechanical, or electrical stimulation. In *O. californica* there was also no detectable difference in emission spectra from tan or brown pigmented bands. The spectra were not fully corrected for system nonlinearities, but the peak and qualitative features such as breadth are reliable.

The green emission of the *in vivo* fluorescence is visually identical to that of the luminescence.

DISCUSSION

The luminescent emission spectrum reported for *O. californica* in this paper has a peak at 510 nm and lacks pronounced shoulders. This differs from the previous report of a peak at 525 nm with a pronounced shoulder at 485 nm (Brehm *et al.*, 1973). These differences in spectra can only be ascribed to variability between animals. Wampler (1977), using as yet unidentified species of ophiuroids, reported a peak at 518 nm with shoulders at approximately 496 nm

TABLE I

Historical findings on luminescent ophuroids.

Species	Sites of luminescence	Color of emission	Source
Acrocnida branchiata	lateral plates	bluish-white	Gotto, 1963
Amphipholis (= *Amphiura*) *squamata*	vertebral muscle	yellow-green	Quatrefages, 1843
	base of tube feet	green	Panceri, 1878
	tube feet	green	Sterzinger, 1907
	lateral plates	yellow-green	Mangold, 1908
	lateral plates	yellow-green	Reichensperger, 1908a, b
	base of spines and lateral plates	—	Trojan, 1909a
	base of spines	—	Buchanan, 1963
Amphiura filiformis	entire spines	green	Mangold, 1907
	entire spines	green	Reichensperger, 1908a, b
	entire spines	—	Buchanan, 1963
Amphiura kandai	—	blue-green	Kato, 1947
Ophiocantha aculeata	lateral plates	blue-green	Herring, 1974
Ophiocantha bidentata (=*spinulosa*)	tube feet and ventral plates	—	Trojan, 1908, 1909b
	spines and lateral plates	yellow-green	Sokolow, 1909
	lateral plates	blue	Herring, 1974
Ophiopsila annulosa	ventral plate, proximal lateral plates, spines, tentacular scales	yellow-green	Mangold, 1907
	ventral plate, spines, proximal lateral plates, tentacular scales	yellow-green	Reichensperger, 1908a, b
	lateral plate, ventral plate, tentacular scales	green	Trojan, 1908, 1909b
Ophiopsila aranea	ventral plate, proximal lateral plates	green	Mangold, 1907
	—	bluish-green	Trojan, 1908, 1909b
	ventral plate, proximal lateral plates	green	Reichensperger, 1908a, b
Ophiopsila californica	ventral plate, lateral plates, spines, tentacular scales, radial shields	green	Brehm *et al.*, 1973
Ophioscolex glacialis	spines		Sokolow, 1909

and 552 nm. Interspecific variation in emission spectra is also suggested by the blue luminescence of *Ophiocantha bidentata* (Herring, 1974). Table I also indicates that blue luminescence may be seen in some other ophiuroids as contrasted with the visually green luminescence of *O. californica* and *A. squamata.* A blue luminescence was also observed in holothuroids and asteroids (Herring, 1974) and certain holothuroids show a peak emission at 472 nm (Wampler, 1977). It would be interesting to investigate the biochemical basis of visually observable differences in emission spectra which exist within the Ophiuroidea and other echinoderms.

In *O. californica* a green fluorescence was found, which is visually identical in color to the luminescent emission. The fact that fluorescence occurs after luminescence suggests that an oxidation product of the reaction is responsible for the fluorescence.

Historically, the statements concerning the distribution and identification of the photocytes in ophiuroids have been contradictory. Table I indicates some of the confusion surrounding the question of which areas within a given ophiuroid are luminescent. In this paper the question over both the cellular origin of luminescence and distribution of photocytes has been resolved in *O. californica* and *A. squamata.* However, the findings for all other species shown in Table I should remain suspect until techniques such as image intensification and fluorescence microscopy have been applied to them.

Fluorescence microscopy has proven to be the most valuable technique for examination of the photocytes in *O. californica* and *A. squamata*. Harvey (1952) reported that in ultraviolet light *A. squamata* and *O. aranea* show a yellowish green fluorescence, but he did not give a description of its distribution, except to note (p. 479) that it was "evenly distributed over the plates and . . . on the feet" in *O. aranea*. He also noted (p. 479) that in *A. squamata* the fluorescence appears as a "network of yellowish green fluorescent material on the plates". Using ultraviolet radiation, we have been able to detect only a weak luminescence-associated fluorescence in both *O. californica* and *A. squamata*. However, such fluorescence can be easily observed in both of these species by exciting with a longer wavelength blue light. This method has also proven successful on many luminescent coelenterates (Morin and Reynolds, 1974). In this paper fluorescence was utilized in conjunction with standard histological techniques to examine individual photocytes. Thus the technique of fluorescence microscopy has allowed identification of both the spatial distribution and morphology of photocytes in these two species of ophiuroids.

The photocytes described here for *O. californica* bear no resemblance to those described by other authors. The varicosities in *O. californica* are much smaller and are more numerous than the photocytes described in any other ophiuroid species. The photocyte processes do not extend to the surface of the spines. Furthermore, the photocytes do not stain with thionin (Reichensperger, 1908b; Buchanan, 1963) or toluidine blue (Buchanan, 1963), as previously reported. We found large gland cells in the spines, which correspond to the cells described by earlier workers. These gland cells neither match the distribution of photogenic tissue nor are they fluorescent. It can be concluded, therefore, that this discussion concerns a newly described cellular origin of luminescence in the genus *Ophiopsila*. The luminescence emanates from varicosities and associated processes and not from a glandular cell type in *O. californica*. A similar morphology is suggested in *A. squamata*. Herring (1974) has found photocytes in the holothuroids and asteroids which may bear similarities to those described here for *O. californica*.

The fact that at the light microscopical level the processes are morphologically indistinguishable from the surrounding nervous tissue is intriguing. Physiologically, it can be demonstrated that propagation of luminescence along the arm is under the direct control of the radial nerve cord (personal observations). These observations suggest the following possibilities. First, the photocyte processes may represent greatly elongated extensions of effector cells which reach to the radial nerve cord. The phenomenon of effector processes approaching axons as a method of innervation in echinoderms has been reported several times. In the tube foot-ampullary system of the asteroid *Astropecten irregularis,* long muscle processes from the ampullary seam extend to internuncial neurons in the radial nerve cord (Cobb, 1967). Also, in the tridentate pedicellaria of the echinoid *Echinus* (Cobb, 1968) and in the vertebral muscles of *Ophiothrix fragilis* (Pentreath and Cottrell, 1971), it has been shown that processes of the muscles pass to the axon bundles for innervation. Secondly, the luminescent processes may be of neural origin. The similar affinity for Holme's silver stain seen in both nervous tissue and photocytes suggests common characteristics. Whether the luminescent tissue represents effector extensions to the nerves or luminescent nerves, it is clear that the histological findings of direct contact between the radial nerve cord and luminescent tissue support the physiological observations of radial nerve cord control.

The authors express thanks to Harold Brehm for drawing Figure 1; to Anne Harrington and Dr. Jon Kastendiek for collecting the specimens of *Ophiopsila californica;* to Dr. George Reynolds for use of the image intensifier and spectrograph; to Sol Gruner, Dr. George Reynolds, and Dr. Alan Walton for their help with the emission spectra data; and to Marlies Natzler for her training in histological techniques.

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SUMMARY

1. The distribution of luminescence in *Ophiopsila californica* and *Amphipholis squamata* is described on the basis of image intensification, fluorescence microscopy, and histological techniques.

2. Luminescence appears to be intracellular.

3. The photogenic cells, termed photocytes, can be identified in histological sections by observation of 460 nm excited fluorescence and appear to have two components: varicosities and processes. The processes are morphologically similar to the neurons of the peripheral nervous system and the radial nerve cord.

4. The theory of gland cells as sites of luminescence in *Ophiopsila* spp. and *Amphipholis squamata* is not supported.

5. The emission spectra of luminescence in *O. californica* and *A. squamata* are broad with a half band width of 71 nm and an approximate emission maximum at 510 nm.

6. Fluorescence appears only after the onset of luminescence.

LITERATURE CITED

BREHM, P., J. G. MORIN, AND G. T. REYNOLDS, 1973. Bioluminescent characteristics of the ophiuroid, *Ophiopsila californica*. *Biol. Bull.*, **145**: 426.

BUCHANAN, J. B., 1963. Mucous secretion within the spines of ophiuroid echinoderms. *Proc. Roy. Soc. London Ser. B*, **141**: 251–259.

CLARK, A. H., 1921. A new Ophiuran of the genus *Ophiopsila* from Southern California. *Proc. Biol. Soc. Wash.*, **34**: 109–110.

CLARK, A. M., 1970. Notes on the family Amphiuridae. *Bull. Brit. Mus. (Natur. Hist.) Zool.*, **19**: 1–81.

COBB, J. L. S., 1967. The innervation of the ampulla of the tube foot in the starfish *Astropecten irregularis*. *Proc. Roy. Soc. London Ser. B*, **168**: 91–99.

COBB, J. L. S., 1968. The fine structure of the pedicellariae of *Echinus esculentus* (L.). I. The innervation of the muscles. *J. Roy. Microsc. Soc.*, **88**: 211–221.

GOTTO, R. V., 1963. Luminescent ophiuroids and associated copepods. *Irish Natur. J.*, **14**: 137–139.

GRUNER, S. M., 1973. An image intensification spectrograph. *Princeton University Technical Report*, **16**: 1–20.

HARVEY, E. N., 1952. Echinodermata. Pages 472–479 in *Bioluminescence*, Academic Press, New York.

Herring, P. J., 1974. New observations on the bioluminescence of echinoderms. *J. Zool. London*, **172**: 410–418.

Kato, K., 1947. Luminous Ophiuroidea. *Zool. Mag. Tokyo*, **57**: 10.

Mangold, E., 1907. Leuchtende Schlangensterne und die Flimmerbewegung bei *Ophiopsila*. *Arch. Ges. Physiol.*, **118**: 613–640.

Mangold, E., 1908. Über das Leuchten und Klettern der Schlangensterne. *Biol. Centrabl.*, **28**: 169–176.

Morin, J. G. and G. T. Reynolds, 1974. The cellular origin of bioluminescence in the colonial hydroid *Obelia*. *Biol. Bull.*, **147**: 397–410.

Panceri, P., 1878. Appendice relativa ad una ofiura luminosa. *Atti. Accad. Sci. Fis. Mat. Napoli*, **7**: 17.

Pentreath, V. and G. Cottrell, 1971. "Giant" neurons and neurosecretion in the hyponeural tissue of *Ophiothrix fragilis* Abildgaard. *J. Exp. Mar. Biol. Ecol.*, **6**: 249–264.

Quatrefages, A. de, 1843. Note sur un nouveau mode de phosphorescence observe chez quelques Annelides et Ophiures. *Ann. Sci. Natur. Zool. Biol. Anim.*, **19**: 183–192.

Reichensperger, A., 1908a. Die Drusengebilde der Ophiuren. *Z. Wiss. Zool.*, **91**: 304–350.

Reichensperger, A., 1908b. Uber das Leuchten von Schlangensternen. *Biol. Centrabl.*, **28**: 166–168.

Reynolds, G. T., 1972. Image intensification applied to biological problems. *Quart. Rev. Biophys.*, **5**: 295–347.

Sokolow, I., 1909. Zür Frage über das Leuchten und die Drusengebilde der Ophiuren. *Biol. Centrabl.*, **29**: 637–648.

Sterzinger, J., 1907. Uber das Leuchtvermögen von *Amphiura squamata* Sars. *Z. Wiss. Zool.*, **88**: 358–384.

Thomas, L. P., 1966. A revision of the tropical American species of *Amphipholis*. *Bull. Mar. Sci.*, **16**: 827–833.

Trojan, E., 1908. Das Leuchten der Schlangensterne. *Biol. Centrabl.*, **28**: 343–352.

Trojan, E., 1909a. Die Lichtenwicklung bei *Amphiura squamata* Sars. *Zool. Anz.*, **34**: 776–781.

Trojan, E., 1909b. Leuchtende *Ophiopsilen*. *Arch. Mikr. Anat. Bd.*, **73**: 883–912.

Wampler, J. E., 1977. Summary of bioluminescence spectra collected during the Southeast Asia Bioluminescence Expedition aboard the *R. V. Alpha Helix* 1975. *Photochem. Photobiol.*, **00**: in press.

Reference: *Biol. Bull.*, **152**: 26–40. (February, 1977)

INTRACELLULAR CRYSTAL-BEARING VESICLES IN THE EPIDERMIS OF SCLERACTINIAN CORALS, *ASTRANGIA DANAE* (AGASSIZ) AND *PORITES PORITES* (PALLAS)

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Initial mineralization of the coral skeleton is thought to occur either in the extracellular milieu or at an intracellular site of calcium carbonate (aragonite) crystallization. As evidence for the former alternative, an organic secretion from the skeletogenic epithelium appears in light microscopic preparations (von Koch, 1882; Duerden, 1904). This secretion is characterized as an acidic mucopolysaccharide (Goreau, 1959) and is amorphous in ultrastructure (Vahl, 1966). It may serve as a template upon which skeletogenesis ensues.

The latter option, an intracellular site of crystal formation (von Heider, 1882; Ogilvie, 1897), recently received support following ultrastructural identification of electron-dense vesicles within the skeletogenic epithelium containing 50 Å threads (Kawaguti and Sato, 1968). Nevertheless, in the absence of critical and definitive evidence for an intracellular site of mineralization, most investigators prefer the extracellular alternative (Vandermeulen and Muscatine, 1973; Vandermeulen, 1975).

Data sufficient to resolve this controversy requires identification of the primordial crystal population itself. The evidence of the site of those crystals would extend previous observations from both extracellular and intracellular supporters and would reassert the significance of skeletogenic tissues during crystal formation in Scleractinian corals (Hayes and Goreau, 1976a). Such were the objectives in this ultrastructural and radiochemical investigation of the Caribbean reef-building hermatype, *Porites porites* (Pallas), and of the North Atlantic ahermatype, *Astrangia danae* (Agassiz).

MATERIALS AND METHODS

Specimens of *Astrangia danae* were collected by dredging in Vineyard Sound off the coast of Woods Hole, Massachusetts, in the United States during the summer of 1975. Water conditions at the collection site at that time were typically 55–70 feet in depth, 18–21° C and 3–5 knot maximum bottom currents. The low illumination, extremely poor visibility and strong water movement made diving with SCUBA impossible. Small rocks encrusted with the coral were transported to the laboratory for maintenance in a running seawater table. For morphological and radiochemical assay, tissues were utilized within one to two days of collection.

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Preparation for microscopy involved the slow addition of 5% glutaraldehyde fixative in filtered seawater (FSW) to yield a final concentration of 2.5% aldehyde. Tissues were fixed for one to two hours at 4° C. Following a series of washes in cold FSW, post-fixation with 1% osmium tetroxide in FSW was conducted. Dehydration of the tissues through graded ethanol solutions continued at 4° C until reaching absolute alcohol. Tissues were then transferred to propylene oxide and embedded in a 50:50 Epon-Araldite resin mixture. The plastic was cured by incubation at 60° C for 48 hours. Silver sections (*ca.* 600 Å thickness) were cut on an LKB Ultratome II for electron microscopy. Sections were viewed unstained or were stained with 7.5 per cent uranyl acetate in 50 per cent ethanol followed by lead citrate. A Philips EM200 was used for ultrastructural observations. Orientation of all tissues was confirmed using Toluidine Blue-stained 1–2 μ thick sections.

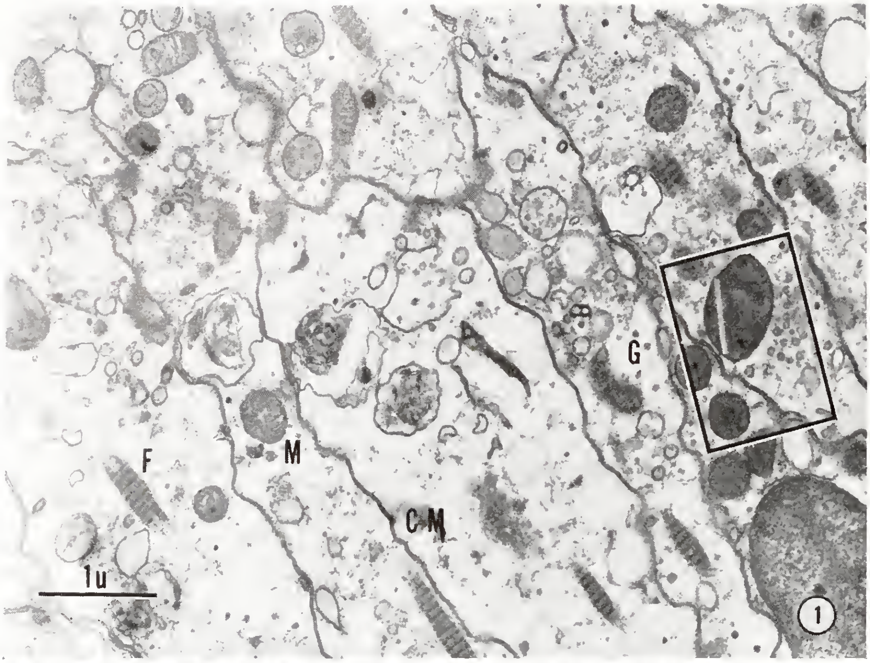
Adult colonies of *Porites porites* were collected from waters adjacent to Port Royal Marine Laboratory (Kingston Harbour Cays) and from the fore-reef near Discovery Bay Marine Laboratory in Jamacia. Water conditions during collection in late winter and spring 1976 were depths of 10–60 feet, temperature averages of 24–26° C and negligible currents. Visibility underwater was excellent, and specimens were collected using SCUBA or snorkel diving techniques. All corals were kept immersed in sea water *en route* to the laboratory.

With the aid of a dissecting microscope, planulae (larvae) were gently separated from the mesenteric tissues within which they develop. Viability of these planulae was established by their swimming behavior. The active larvae were transferred to a finger bowl containing FSW and further selected on the basis of vitality and morphological integrity for transfer to small aquaria for settlement experiments. Substrates provided for larval attachment included clean glass microscope slides and thin slices of bleached, cleaned and salt water-equilibrated skeletons of *Porites.* Aquaria with planulae were equipped with either a capillary pipette or a hypodermic needle as an aerator port, and a slow discharge of bubbles was regulated so as not to disrupt larval adhesions.

Once attachment was firmly secured, the larvae were removed and fixed either with 2.5% glutaraldehyde or 1.0% osmium tetroxide in FSW at 4° C. Free-swimming planulae and planulae attached for periods of less than one day through one week were preserved for morphological study. Procedures followed duplicate exactly those described above for *Astrangia* adults, with the exception that electron microscopy was accomplished using an AEI instrument.

Sections of all plastic-embedded coral tissues were cut with a Dupont diamond knife. In an attempt to preserve normal morphological relationships, no effort was made to decalcify specimens. For free-swimming planulae, this was not a concern; for attached planulae and adults, tissues were embedded with skeletal substrates and skeletons intact. Blocks of embedded tissues were faced and trimmed leaving small portions of the skeletal matrix for orientation both within the block itself and within thick and thin sections. The presence of this calcified material provided an opportunity to investigate the ultrastructure of the skeleton in ultrathin section.

Several experiments were conducted to determine incorporation of radioactive ^{45}Ca by adult *Astrangia danae.* Aerated sea water, filtered through a Nalgene filter unit with a 0.2 μ membrane, was prepared with 1.0 μCi/ml of $^{45}CaCl_2$ (New England Nuclear). Volumes of 200 ml were dispensed into clean glass finger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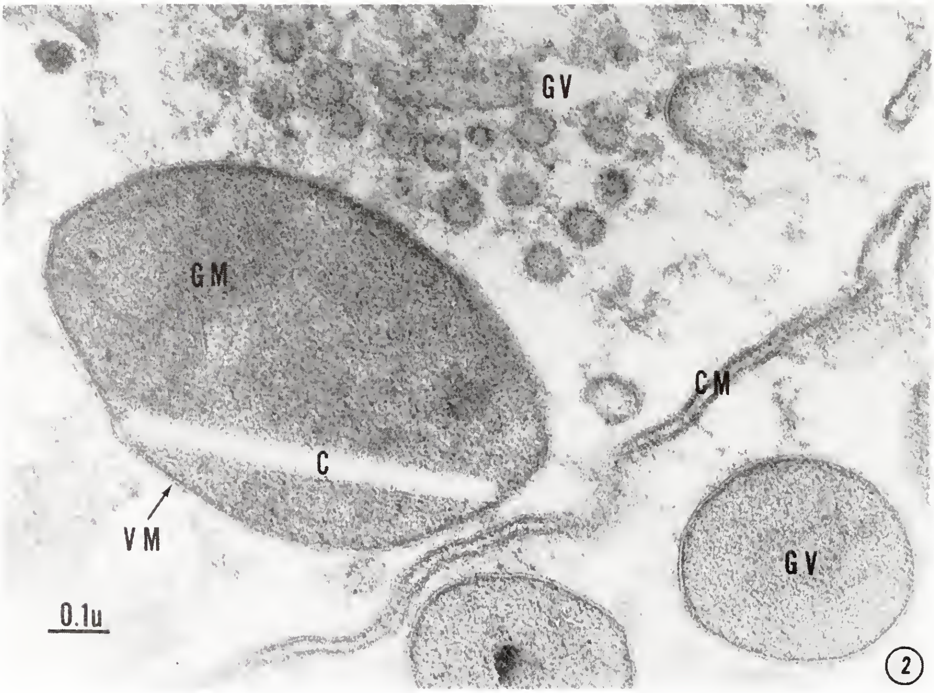
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bowls and small colonial growths of *Astrangia* were added. Skeletal weights of these colonies did not exceed 0.1 g. Temperature was maintained at 24° C by setting the aquaria in a seawater table; aeration was constant.

Following exposure to radioactive sea water, corals were rinsed at least five times in cold filtered sea water. Each was wrapped in parafilm envelopes and frozen to terminate metabolic activity. Further analysis consisted of extracting tissue from the skeleton by treatment with concentrated ammonium hydroxide (14.7 M). Skeletons were rinsed two to three times to complete release of tissue. The skeletal fraction was then solubilized in 1.5 N hydrochloric acid and dried in an oven.

The soft tissue fraction in basic solution consisted of the original supernatant to which skeletal washings were added. This was allowed to settle, and the liquid fraction removed and evaporated in an oven. The basic residue was treated with 1.5 N HCl to extract the acid soluble fraction which was decanted and saved. The insoluble residue was then added to the original basic supernatant to integrate the basic tissue fraction. Summarily, three compartments of the original coral were separated for incorporation analysis: a basic tissue fraction, an acid tissue fraction and a skeletal fraction. Each of these was dried to a powder and counted in a Nuclear-Chicago planchet scintillation counter.

RESULTS

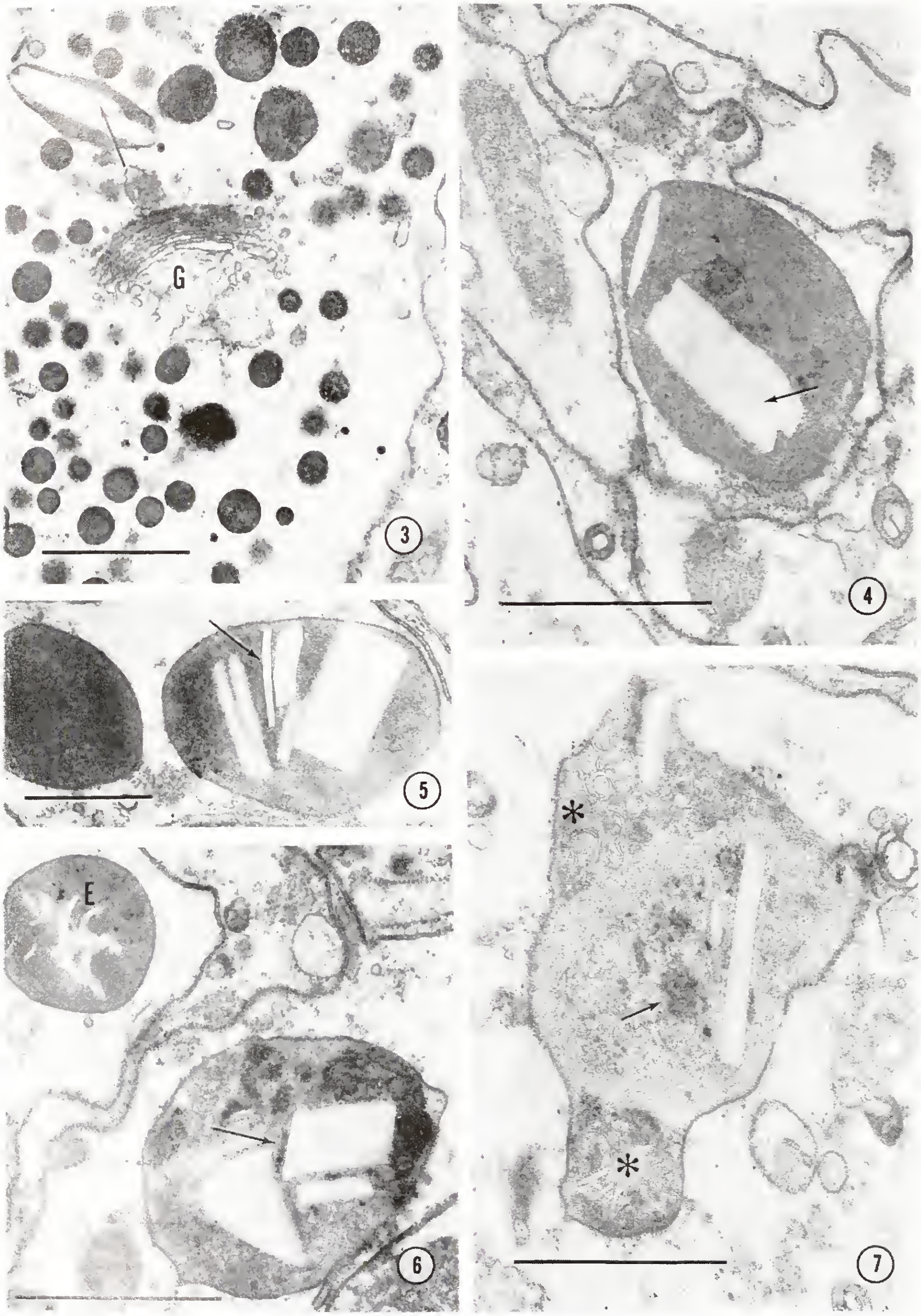
Light microscopy

The body wall of the free-swimming planula of *Porites* comprises two epithelia cemented and supported by a thin connective tissue lamina or mesoglea. The innermost epithelium or gastrodermis is low cuboidal and is easily distinguished by large dense cytoplasmic zymogen granules. This epithelial surface lines the coelenteron or digestive cavity and throws itself into a complex of mesenterial septae, all involved in nourishing the organism. The gastrodermis is also the residence of zooxanthellae or symbiotic algae in hermatypic corals.

The superficial epithelium or epidermis is high pseudo-stratified columnar in all regions, except in surfaces of attachment of the coral organism to a solid substrate; whereas the free-swimming larva has only a pseudo-stratified columnar epithelium, morphosis of that epidermis occurs upon settlement, and the resulting epithelium assumes a squamous cellular configuration which may or may not be stratified. Because of its juxtaposition to sites of the coral skeleton, this transformed squamous epithelium is termed the skeletogenic or "calicoblastic" epidermis. Epidermal differentiation into a free columnar zone and a skeletogenic squamous zone persists in the settled, attached planula of *Porites* and in the adults of *Porites* and *Astrangia*. These epidermal zones are morphologically contiguous through a transitional zone at the edge of the organism, if solitary, or at the edge of the colony, if colonial.

FIGURE 1. Apical cytoplasm of epidermal epithelium of adult *Astrangia danae*. One crystal-bearing vesicle appears within insert. Other cytoplasmic organelles include mitochondria (M), flagellar axial filaments (F), glycogen (G) and lateral cell membranes (CM).

FIGURE 2. Enlargement of crystal-bearing vesicle in Figure 1 insert, revealing detail of trilaminar membrane (VM), granular matrix (GM) and crystal rodlet (C) along with several Golgi vesicles without crystals (GV). Note similarity in matrix compared with other vesicles.



FIGURES 3-7. Assortment of crystal-bearing vesicles in *Astrangia danae*. Scale bars are 1 μ.

The principal cell type in the free epidermis is a columnar cell with a single flagellum at its apical surface and a striated border. Other cell types recognized are nematocytes, mucous goblet cells and granular cells. These cells persist in the skeletogenic epidermis although in remodelling during settlement, the apical specialization of the principal cell type, including the flagellary apparatus, disappears.

Electron microscopy

Ultrastructural examination of the supranuclear region of the epidermal cell reveals numerous assorted cytoplasmic organelles. Richly endowed with spherulitic mitochondria and displaying pronounced vacuoles and glycogen rosettes, this cytoplasmic zone also accommodates the Golgi membrane system of flattened sacs and assorted dense granular vesicles (Figs. 1, 8).

Within the larger vesicles, electron-lucent crystalline configurations appear (Figs. 1–12). The location and structure of these crystal-bearing vesicles indicates that they are derived from the Golgi system. Measurements on the crystals establish that they are flattened plates or rodlets with maximum dimensions of 0.7 μ, by 0.1 μ by 0.3 μ in both the adult *Astrangia* and the larval *Porites*. Crystallographic description of these structures with three unequal axes at right angles to each other conforms to the orthorhombic configuration. Such geometry is characteristic of the aragonitic polymorph of calcium carbonate (cf. Palache, Berman and Frondel, 1944). Further analysis of these crystals is in progress to confirm the lattice configuration which is expected to be that of aragonite.

In many of these vesicles, multiple crystalline profiles are observed, and some appear coupled or closely linked together (Figs. 3–5). Again, in a crystallographic context, this resembles a twinning phenomenon, a common occurrence in aragonite (cf. Palache *et al.*, 1944).

From their appearance in ultrathin plastic sections, it is concluded that these crystalline inclusions are electron-lucent, that is, freely penetrable by the electron beam in 600 Å plastic sections. The crystalline zones reveal a subtle texture and compare favorably with the electron density of the background cytoplasmic matrix. Examination of adult coral skeletons, coral substrates used for larval attachment and settled larval primary skeletons establishes electron-lucency of aragonite crystals in thin sections viewed with the electron microscope. In this technical procedure, the crystals are not solubilized during tissue preparation, and the analysis of unstained sections containing crystals eliminates the possibility of crystal removal following exposure to uranyl acetate as reported in the literature (Wilbur, Colinvaux and Watabe, 1969).

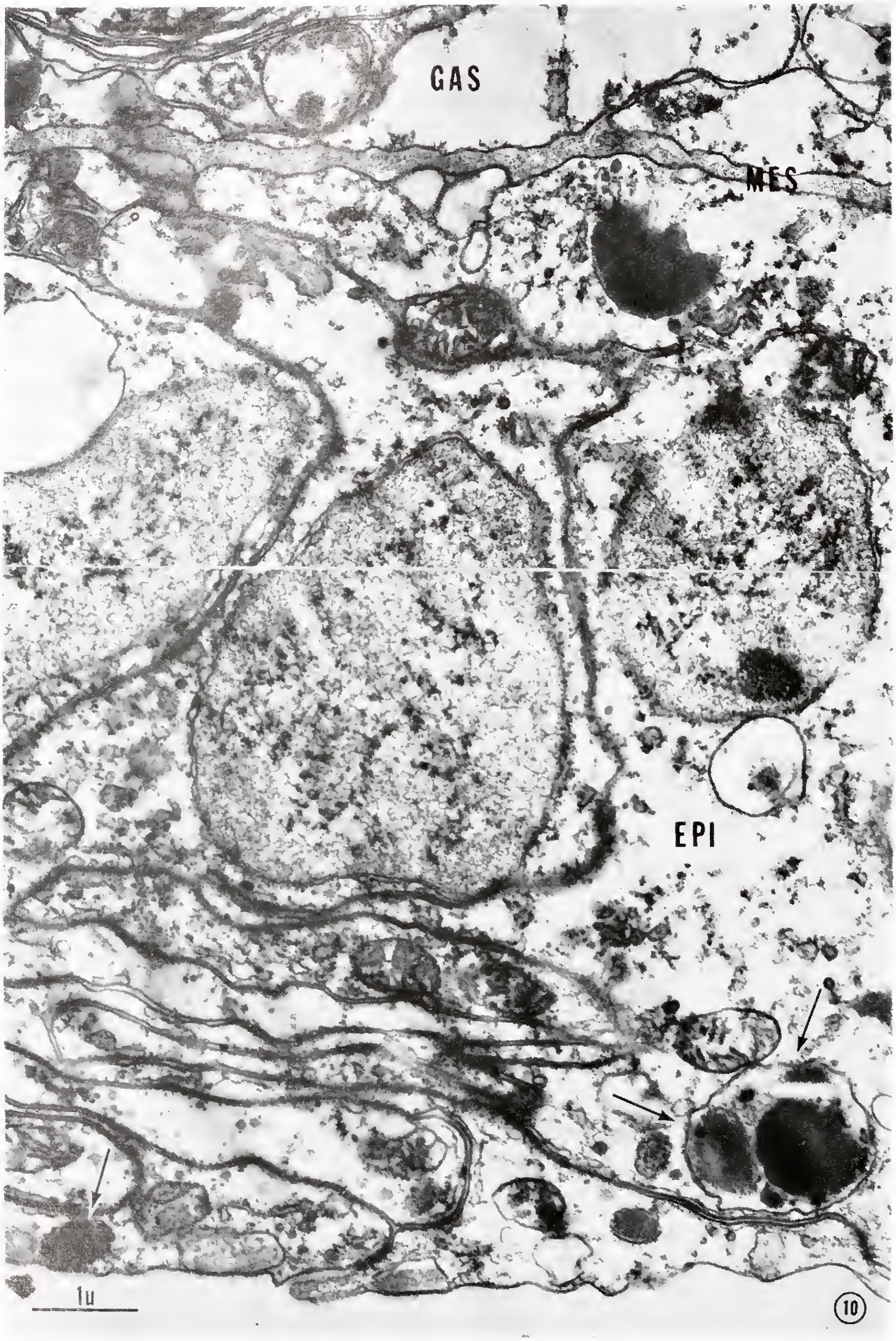
FIGURE 3. Twinning configuration of crystals (arrow) with thin sheet of matrix between units. Note proximity to and structural similarity with Golgi membrane system (G).

FIGURE 4. Multiple crystals within one vesicle. Note twinning of crystals (arrows).

FIGURE 5. Crystal-bearing vesicle revealing condensation of matrix substance around crystal surface (arrow).

FIGURE 6. Organization of matrix constituents into myelinised configuration at crystal surface (arrow). Crystal embryo (E) in vesicle.

FIGURE 7. Bizarre configuration of swirling membrane-like profiles (asterisk) and heterogeneity of matrix substances (arrow) in crystal-bearing vesicle.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the matrix substance and the osmiophilic quality of that material. Identification of the vesicles themselves in light microscopy is uncomplicated since they average 1.5 μ in diameter and are easily resolvable. Furthermore, the vesicles may be stained in thick sections with routine basophilic dyes such as Toluidine blue or Methylene blue.

In the free epidermis, there is extensive development of the apical cytoskeletal complex consisting of desmosomal tonofilaments, the terminal web of cytoplasmic fibrils and lateral extensions from the flagellar axial fibers (Hayes and Goreau, 1976b). The absence of these specializations in the skeletogenic epithelium would allow crystal release at that site (Figures 10–12). The granular matrix of the crystal-bearing vesicle, as well as the matrix material of vesicles bearing no crystals, contribute to the formation of the organic phase of the exoskeleton. Following release from the cell by exocytosis this matrix material would provide an important, perhaps the only, source of calcium for continued crystal growth and nucleation.

Incorporation of radioactive ^{45}Ca

The soft tissues of *Astrangia danae* incorporate ionic ^{45}Ca rapidly as shown in Table I. More than 90% of that radioactivity is extractable in the basic-soluble tissue fraction. The acid-soluble tissue fraction, which can be interpreted as representing ^{45}Ca bound into acid-soluble protein and/or into crystals of acid-soluble calcium carbonate (cf. Palache *et al.*, 1944), is derived from the cytoplasm and contributes a small percentage of total incorporated radio-activity, never exceeding 5%. Skeletal incorporation in six hours or less constitutes the smallest fraction of the radio-calcium pool at 3% or less of total uptake.

DISCUSSION

Ultrastructural data from epidermal cells of Scleractinian corals, supported by radiochemical assay, substantiate the presence of a crystalline structure which resembles the aragonitic polymorph of calcium carbonate. This intracellular crystalline product is demonstrable following preparation of tissues for electron microscopy, as well as in unfixed and treated samples from ^{45}Ca uptake studies. Organization of the organic matrix of the crystal-bearing vesicle evidences the existence of crystalline inclusions *in vivo* prior to fixation or subsequent processing of the tissue. The preparative techniques used in this investigation are similar to procedures employed by other investigators of coral ultrastructure (Kawaguti, 1964; Kawaguti and Sato, 1968; Vandermeulen, 1974, 1975; Vahl, 1966) in which crystalline inclusions are denied.

The question of electron-lucency of the aragonite crystal has led to considerable investigation on our part as to the appearance of calcium crystals in transmission electron microscopy. Reports in the literature of mineralized systems composed of aragonite have indicated these calcium crystals as electron-lucent (Vander-

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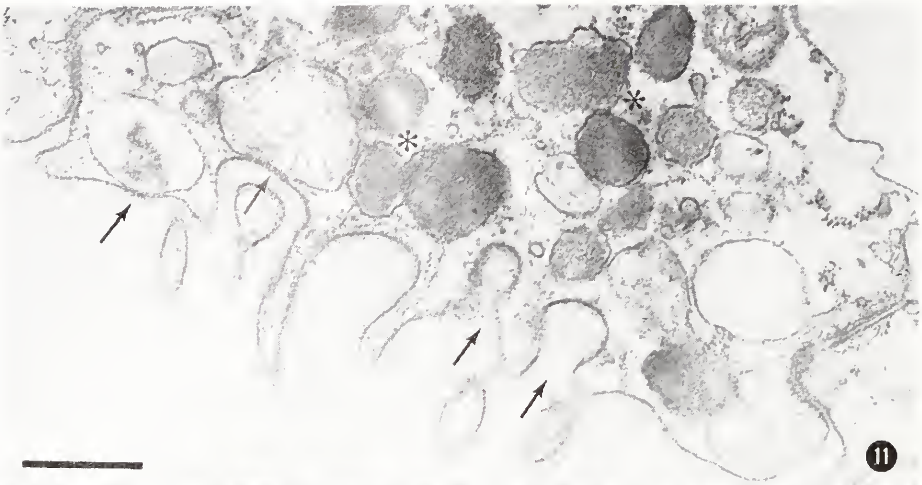
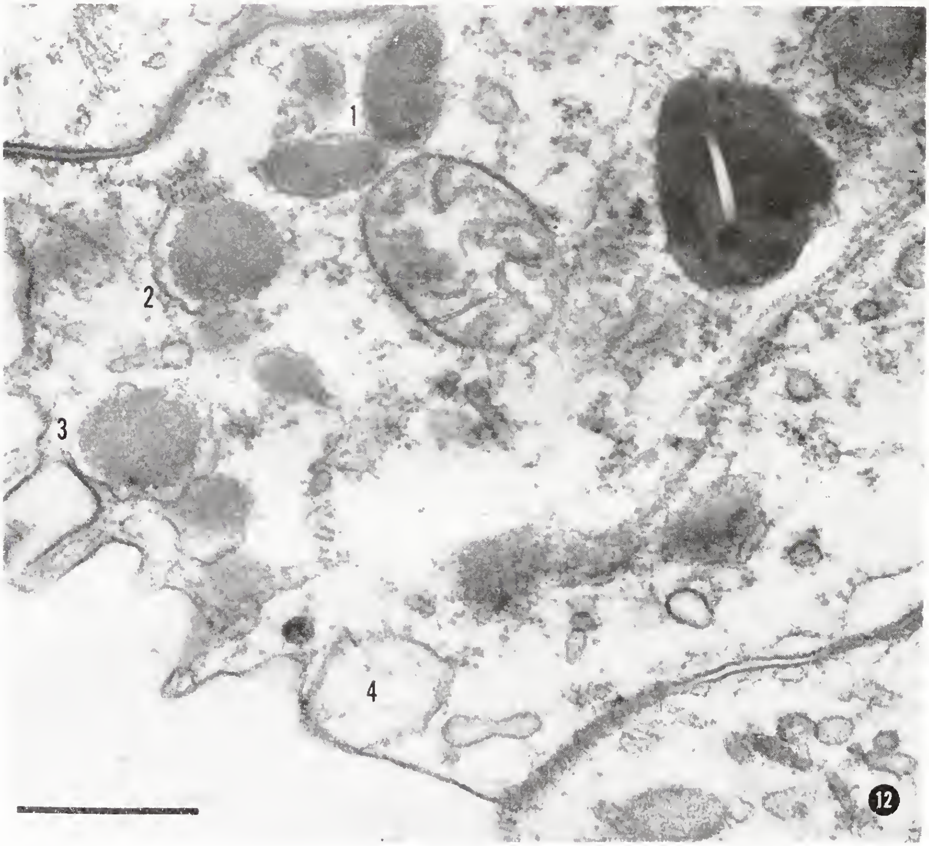
FIGURE 10. Montage of attached epidermal epithelium in the planula, *Porites porites*, showing localization of crystal-bearing vesicle (arrows) along surface of contact of organism with its substratum. A vesicle without crystals is being released in lower left of field (single arrow). Epidermis (EPI), mesoglea (MES) and gastrodermis (GAS) as designated for orientation. Compare surface appearance with Figure 8.





FIGURES 11 and 12. Attached surfaces of epidermis of *Porites porites* evidencing release of contents of Golgi vesicles by exocytosis. Scale bars are 0.5 μ.

TABLE I

Incorporation of ^{45}Ca *by adult* Astrangia danae.

Exposure	Fraction					
	NH$_4$OH tissue extract		HCl tissue extract		HCl skeletal extract	
	cpm	% total cpm	cpm	% total cpm	cpm	% total cpm
20 min	40,626	94.2	1,340	3.1	1,165	2.7
2 hr	57,406	92.9	2,501	4.0	1,972	3.1
6 hr	89,023	92.8	4,453	4.6	2,583	2.6

meulen and Watabe, 1973), as well as electron-dense (Wilbur *et al.*, 1969). Another author (Anderson, 1973) has categorically stated that calcium is electron-dense in transmission microscopy. Our experience has been that the section thickness is critical for determination of electron penetrability. Sections with silver-gray interference color reveal electron-lucent crystals in the vesicles of adherent coral planulae and adult polyps.

Identification of an intracellular crystalline product contributed by the epidermal cells to the site of skeleton formation prompts emergence of a novel concept of cellular regulation of events culminating in coral skeletogenesis. Mechanisms by which cells of the organism dictate specific construction or design of their skeleton must now encompass regulatory roles for the vesicle membrane, the plasmalemma, the protein synthetic machinery (rough endoplasmic reticulum), the packaging system (Golgi membranes, agranular reticulum) and, in general, the entire physico-chemical character of the apical cell cytoplasm.

The membrane-bound Golgi vesicle serves as a miniature crystal fabrication station as well as a vehicle for transport of the crystal to an appropriate site for discharge. Release of the crystal, embedded within an organic matrix and endowed with a pre-established affinity for ionic calcium and a charge of lipo-protein-bound calcium, allows continued calcification extracellularly. The charged organic matrix is especially important since skeletal exchange of calcium with the environment is improbable (Goreau and Goreau, 1960). Microprobe analysis of cytoplasmic vesicles without crystals in another reef-building coral has established that they are a site of accumulation of calcium (Vandermeulen, 1975). The vesicular membrane gains significance as a site of regulation of calcium ion transport and of generation of carbonate ion perhaps through enzymatic action of carbonic anhydrase as suggested by Goreau and Goreau (1960). The phosphatase

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FIGURE 11. Vesicles in cell cytoplasm become confluent with one another (asterisks), fuse with plasma membrane and discharge contents as vesicle membrane is integrated into cell surface (arrows).

FIGURE 12. Assortment of vesicles, one crystal-bearing, at various stages of release of contents by exocytosis. Confluence of vesicles (1), clear zone developed around granular matrix (2), near-fusion between vesicle membrane and plasmalemma (3) and membrane fusion with discharge of vesicle contents (4).

enzymes, especially the alkaline phosphatases, if localized in or on the vesicle membrane, may exert inhibitory influence over crystal formation as suggested by Simkiss (1964).

The physical and chemical status of the apical cell cytoplasm is important with respect to transport and release of the crystalline product. For release into the extracellular milieu contact between the vesicle membrane and the plasmalemma must be established. Beyond that, however, nucleation of the intracellular crystal itself may be dependent upon the environment of the vesicle. Two of the most critical influences upon biological crystallization are pH and ion strength (Bachra, 1973). Abrupt alteration of either or both of these factors with settling of the coral planula could trigger $CaCO_3$ clusters or crystal embryos which spontaneously grow into crystals within the calcium-loaded vesicles.

The observation that crystal generation encompasses a larger area of epidermis than just the substrate-contacted surface indicates that changes within the epidermis do not relate to the morphological transformation at that site. Upon settling, cessation of locomotory function by the flagellae may provide the stimulus for local responses in the apical cell cytoplasm inducing crystal formation uniformly throughout the epithelium. That the entire epidermis of the planula with the exception of the juxta-stomodeal epithelium would be competent to generate aragonitic crystals seems logical since the planula has no control over the size, contour or consistency of its settlement site. Whatever the orientation of the planula upon settlement, or whatever the extent of the substratum, crystal-bearing epidermis is available for establishment of the skeleton.

Calcium-accumulating vesicles within the intracellular matrix of vertebrate ossification systems have revealed needle-like crystalline structures identified as apatite (Anderson, 1969; Bernard and Pease, 1969; Bonucci, 1970). These vesicles appear to be the site of initial deposition of calcium phosphate crystals in cartilage, bone and dentin. As early as the seventh day of development of the chick femur, mineralized vesicles have been sighted confirming their physiological significance in embryogenesis. Although the vertebrate vesicles might be derived from chondrocytes, they have only been observed as independent extracellular structures in vertebrate tissues (Anderson, 1973). In contrast to the intercellular vesicles in vertebrate systems, those described in Scleractinia in this study are unquestionably intracellular.

The granular matrix material of the vesicles remains uncharacterized in both vertebrate and invertebrate systems. However, as originally proposed by Anderson (1967) the osmiophilic character of the material strongly suggests that it is lipidic. The observation in Scleractinia of myelinized configurations parallel to the crystal surface suggests that this material is lipo-protein and that it is capable of spontaneous organization along an appropriate interface into trilaminar membranous profiles.

It is important to note that the crystalline structures which have been described in this study in corals are not interpreted as precursor substances nor as building blocks to be utilized in skeleton formation. The process by which the coral skeleton is generated is envisioned as a process of nucleation which utilizes seed crystals liberated from the epithelial cell to initiate skeletogenesis. Once the seed is deposited extracellularly, principles governing crystal growth in a supersaturated solution may prevail. Extracellular mineralization proceeds with the initial crystal serving as a nucleation catalyst for formation of other crystals by epitaxy. The organic matrix which is liberated along with the seed crystal provides an intrinsic supply of ionic calcium. The organic matrix is eventually engulfed by the mineral product thereby limiting the skeletal growth consequent to one episode of intracellular crystal release. The free-swimming larvae possess granular vesicles, but crystals appear only after the decumbent organism has become adherent and is engaged in skeletogenic function. The adult coral gives evidence that this process is spontaneously continued throughout the life of the organism and supports the interpretation that the skeletogenic potential of the single crystal and its organic matrix is self-limited.

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SUMMARY

Orthorhombic aragonitic crystals, embedded with a granular lipo-protein matrix and surrounded by a trilaminar membrane, are localized in the apical cytoplasm of epidermal cells of Scleractinian corals. Adult specimens of *Astrangia danae* (Agassiz) and settled planulae of *Porites porites* (Pallas) contain crystals averaging 0.7 μ by 0.1 μ by 0.3 μ within Golgi-derived vesicles. Short-term labelling with ^{45}Ca reveals distribution of radioactivity among a basic tissue fraction (92%), an acid tissue fraction (5%) and a skeletal fraction (3%).

Identification of the primordial crystal population within membrane-bound vesicles provides overwhelming evidence for the intracellular mode of calcification in Scleractinia. Moreover, it permits development of a novel concept of cellular regulation over these dynamic events. The membrane-bound vesicle is a miniature crystal fabrication station and a vehicle responsible for transportation of seed crystals and an organic matrix material to sites of discharge from the cell. The vesicle membrane becomes a probable locus of active transport and enzymatic activity as well as a physical barrier to be penetrated for release of vesicle contents into the extracellular milieu. Contact between the vesicle membrane and the plasmalemma would result in exocytosis and the onset of skeletogenesis. Principles governing crystal growth would prevail from then on. The released crystal becomes a nucleation catalyst and the organic matrix, a supply of ionic calcium for self-limiting crystallization. Crystals are produced by the organism spontaneously and continuously from shortly after larval attachment throughout the life of the polyp. Therefore, these membrane-bound vesicles signal the dynamic process by which initiation, differentiation, growth and limitation of the coral skeleton is regulated.

LITERATURE CITED

Anderson, H. C., 1967. An electron microscopic study of induced cartilage development and calcification. *J. Cell Biol.*, **35**: 81–101.

Anderson, H. C., 1969. Vesicles associated with calcification in the matrix of epiphyseal cartilage. *J. Cell Biol.*, **41**: 59–72.

Anderson, H. C., 1973. Calcium-accumulating vesicles in the intercellular matrix of bone. *Ciba Found. Symp.*, **11**: 213–246.

Bachra, B. N., 1973. Nucleation in biological systems. Pages 845–881 in I. Ziplan, Ed., *Biological mineralization.* John Wiley, New York.

Bernard, G. W., and D. C. Pease, 1969. An electron microscopic study of initial intramembranous osteogenesis. *Amer. J. Anat.*, **125**: 271–290.

Bonucci, E., 1970. Fine structure and histochemistry of calcifying globules in epiphyseal cartilage. *Z. Zellforsch. Mikrosk. Anat.*, **103**: 192–217.

Duerden, J. E., 1904. Recent results on the morphology and development of coral polyps. *Smithson. Misc. Collect.* **47**: 93–111.

Goreau, T. F., 1959. The physiology of skeleton formation in corals. I. A method for measuring the rate of calcium deposition by corals under different conditions. *Biol. Bull.*, **116**: 59–75.

Goreau, T. F. and N. I. Goreau, 1960. The physiology of skeleton formation in corals. IV. On isotopic equilibrium exchanges of calcium between corallum and environment in living and dead reef-building corals. *Biol. Bull.*, **119**: 416–427.

Hayes, R. L. and N. I. Goreau, 1976a. Cellular mechanisms regulating coral skeletogenesis. *J. Cell Biol.*, **70**: 48B.

Hayes, R. L. and N. I. Goreau, 1976b. The epidermal flagellary apparatus in the larvae of *Porites porites* (Scleractinia). *Proc. Ass. Island Mar. Lab. Caribbean,* **12**: 31.

Kawaguti, S., 1964. Electron microscopy on the spicules and the polyp of a gorgonian, *Euplexaura erecta. Biol. J. Okayama Univ.*, **10**: 23–38.

Kawaguti, S. and K. Sato, 1968. Electron microscopy on the polyp of staghorn corals with special reference to its skeleton formation. *Biol. J. Okayama Univ.*, **14**: 87–98.

Ogilvie, M. M., 1897. Microscopic and systematic study of madreporarian types of corals. *Phil. Trans. Roy. Soc. London Ser. B*, **187**: 83–345.

Palache, C., H. Berman and C. Frondel, 1944. Pages 182–193 in *Dana's system of minerology*, 7th Ed. J. Wiley, New York.

Simkiss, K., 1964. Phosphates as crystal poisons of calcification. *Biol. Rev.*, **39**: 487–505.

Vahl, J., 1966. Sublichtmikroskopische Untersuchuingen der Kristallinen Grundbauelemente und der Matrixbeziehung zwischen Weichkorper und Skelett an *Caryophyllia* Lamark 1801. *Z. Morphol. Tiere*, **56**: 21–38.

Vandermeulen, J. H., 1974. Studies on reef corals. II. Fine structure of planktonic planula larva of *Pocillopora damicornis* with emphasis on the aboral epidermis. *Mar. Biol.,* **27**: 239–249.

Vandermeulen, J. H., 1975. Studies on reef corals. III. Fine structural changes of calicoblast cells in *Pocillopora damicornis* during settling and calcification. *Mar. Biol.,* **31**: 69–77.

Vandermeulen, J. H. and L. Muscatine, 1973. Influence of symbiotic algae on calcification in reef coral: critique and progress report. Pages 1–19 in W. B. Vernberg and F. J. Vernberg, Eds., *Symbiosis in the sea, Volume II.* University South Carolina Press, Columbia.

Vandermeulen, J. H. and N. Watabe, 1973. Studies on reef corals. I. Skeleton formation by newly settled planula larva of *Pocillopora damicornis. Mar. Biol.*, **23**: 47–57.

von Heider, A., 1881. Die Gattung Cladocora Ehrenberg. *Sitzungsber. K. Akad. Wiss.*, **84**: 634–667.

von Koch, G., 1882. Uber die Entwicklung des Kalkskelettes von *Asteroides calycularis* und dessen morphologische Bedeutung. *Mitt. Zool. Sta. Neapel*, **3**: 284–292.

Wilbur, K. M., L. H. Colinvaux and N. Watabe, 1969. Electron microscope study of calcification in the alga *Halimeda. Phycologia,* **8**: 27–35.

Reference: *Biol. Bull.,* 152: 41–50. (February, 1977)

GENIC SIMILARITY OF AMERICAN AND EUROPEAN SPECIES OF THE LOBSTER *HOMARUS*

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In the course of investigating the population genetics of American lobsters (*Homarus americanus* M-E) and its implications for fisheries management and aquaculture, a survey of electrophoretically detectable protein variation was conducted (Tracey, Nelson, Hedgecock, Shleser and Pressick, 1975). This study revealed both a low level of genetic variation within populations relative to other invertebrates, and little genetic differentiation between populations.

In order to evaluate the potential of hybridization as a breeding program, it became important to examine genetic variation in the European lobster, *H. gammarus* (L), and to determine the degree of protein divergence between these two species of the genus. The results of this study are reported here.

The low level of protein divergence which was found, when contrasted with the morphological distinctness of the two species at all stages from egg to adult, has a bearing on the notion that protein evolution and biological (or organismal) evolution may proceed relatively independently of one another, at quite different rates (see, for example, King and Wilson, 1975).

MATERIALS AND METHODS

Samples of *H. gammarus* from five localities were as follows (from North to South): 1) HGN—ten ovigerous females and ten males from Hordaland (Bergen), Norway, June, 1975; 2) HGS—four females and four males from Iona, Scotland, November, 1975; 3) HGI—six ovigerous and three nonovigerous females from various inshore localities, Ireland, October, 1975; 4) HGZ—eleven females and nine males collected 40 km south of Holyhead, Wales, in the Bay of Caernarvon, January, 1975; 5) HGW—one female and four males from the area of Aberdaron, Wales, November, 1975. Carapace lengths of these specimens ranged from about 85 to 118 mm.

Tissue preparation and electrophoretic techniques were the same as described for *H. americanus* (Tracey *et al.,* 1975). Muscle, heart, gonad, hepatopancreas, gill and green gland tissues were taken from all HGZ specimens and from seven HGN males and one HGN female. As the remaining individuals were needed for breeding purposes, only a single pereiopod was removed from each for electrophoretic analysis. Enzymes assayed in these leg tissue extracts (with numerical suffixes designating the loci scored on zymograms showing multiple zones of activity) were: esterase (-2, -8), fumarase (-1, -2), glyceraldehyde-3-phosphate dehydrogenase, hexokinase (-2, -3), malate dehydrogenase (-1, -2), 6-phosphogluconate dehydrogenase, phosphoglucose isomerase (-3, -4), phosphoglucomutase (-1), tetrazolium oxidase (-1, -2, -3, -5), and triosephosphate isomerase. In addition, general proteins (-1, -2, -3, -4, -5), were revealed by the coomassie blue staining method.

After eye spots had developed in the egg masses of the ovigerous HGN and HGI females, egg samples were screened for protein variation as described previously for larval and juvenile stages (Hedgecock, Nelson, Shleser and Tracey, 1975). Due to the small amount of tissue in a lobster egg, it was necessary to collect two subsamples (eight to twelve eggs each) from each female in order to complete initial assays. The following enzymes were assayed in these progenies: esterase (-2, -4, -5, -6b, -8), fumarase (-1, -2), glutamate-oxaloacetic transaminase, glyceraldehyde-3-phosphate dehydrogenase (-1, -2), 6-phosphogluconate dehydrogenase, phosphoglucose isomerase (-3, -4), phosphoglucomutase (-1), tetrazolium oxidase (-2, -4, -5), and triosephosphate isomerase. Only the dark-staining protein -2 zone is routinely detected in egg samples. *Est-6b*, detected in egg, larval and juvenile whole animal extracts, is polymorphic in *H. americanus* (Hedgecock, Shleser and Nelson, 1976 and unpublished data). *H. gammarus* progenies surveyed here appear to be monomorphic for *Est-6b*^{100}, but due to the small sample size this locus is not included in calculations of genetic distance.

Tissue samples from three offshore American lobsters (GBS sample, Tracey *et al.*, 1975) were electrophoresed along with European samples in order to determine allozyme identities in the two species. Because of small sample sizes and lack of gene frequency differentiation, the four Irish Sea samples (HGS, HGI, HGZ, HGW) are pooled in the analysis.

Using Nei's statistics of genetic identity and genetic distance (Nei, 1972), pairwise comparisons between the Norway and Irish Sea samples and between these samples and eight *H. americanus* population samples studied previously are made for each locus and averaged.

RESULTS

Seventeen of eighteen proteins assayed in *H. americanus* are demonstrable in *H. gammarus;* no activity is detected by the assay for leucine aminopeptidase. Zymograms for these proteins comprise a total of forty-one zones of staining activity that are either identical to or homologous with the zones described for *H. americanus.* Gene loci are symbolized by italicized abbreviations of enzyme names with numerical suffixes distinguishing among the multiple gene products detected on several zymograms (for a list of these see Tracey *et al.*, 1975). Alleles encoding the most common *H. americanus* allozymes in each zone are arbitrarily designated 100. *H. gammarus* allozymes and alleles are assigned numerals obtained by adding to or subtracting from 100 the number of millimeters separating a given allozyme from the common *H. americanus* allozyme after routine electrophoresis.

Genetic variability in H. gammarus *populations*

Full complements of nearly forty loci were studied only in eight Norway and twenty Irish Sea (HGZ) lobsters sacrificed for that purpose. Sample sizes for most loci were augmented by analyses of pereiopod tissues from additional adults reserved for broodstock and by estimation of paternal genotypes from progeny analyses. Thus, maximum numbers of independent genomes studied at some loci in the Norway and Irish Sea samples are 60 and 96, respectively. Due to the variety of available sample material, however, average sample size per locus is considerably less than maximum—39 ± 3 genomes in the Norway sample and 57 ± 4 in the Irish Sea sample (Table I). Nevertheless, these samples are comparable to our previous *H. americanus* samples both with respect to numbers of genomes and, more importantly, for purposes of estimating genic heterozygosity and genetic distance (Nei and Roychoudhury, 1974), with respect to numbers of loci.

The amounts of genetic variability within the Norway and Irish Sea *H. gammarus* populations are essentially the same as those in *H. americanus.* Three measures of variability (average number of alleles per locus, proportions of polymorphic loci, and average proportions of heterozygotes per locus) agree closely with the *H. americanus* values (Table I).

In different *H. americanus* populations four loci, *Est-2, Pgi-4, Pgm-1* and *Tpi,* show a maximum of three alleles per locus; the remaining polymorphisms are diallelic. This is likewise true of variation in *H. gammarus. Me* in the Irish Sea population sample is the sole triallelic polymorphism, and the average number of alleles per locus in *Homarus* populations is thus consistently 1.2.

Two measures of the proportions of polymorphic loci per population are also consistent in *Homarus* species. P, the proportion of loci showing any allozyme variation, is 20 ± 1%, and the more restrictive $P_{0.95}$ (see Table I footnote) is 14 ± 1%.

Finally, observed average heterozygosity (per locus or per individual) is 3.3% in Norway and 5.5% in Irish Sea populations. These rank lowest and ninth, respectively, among ten *Homarus* populations studied; thus, mean heterozygosity

TABLE I

Summary statistics of genetic variation in the European and American species of Homarus. *See text for detailed explanation of the Norway and Irish Sea population samples. Data for* H. americanus *are averages over eight population samples studied previously* (*Tracey* et al., *1975*).

	H. gammarus		
	Norway	Irish Sea	*H. americanus*
Number of loci studied	39	37	37.4 ± 1.8
Average number of genomes sampled per locus	39 ± 3	57 ± 4	59 ± 12
Average number of alleles per locus	1.20 ± 0.07	1.22 ± 0.08	1.23 ± 0.03
Proportions of polymorphic loci per population			
P*	0.205	0.189	0.209 ± 0.025
$P_{.95}$**	0.154	0.108	0.141 ± 0.015
Average proportions of heterozygotes per locus			
Observed (H_o)	0.033 ± 0.013	0.055 ± 0.025	0.039 ± 0.005
Expected (H_e)	0.040 ± 0.016	0.058 ± 0.026	0.046 ± 0.006

* P is the proportion of loci at which two or more alleles are detected.

** $P_{0.95}$ is the proportion of loci at which the most common allele has a frequency no greater than 0.95.

in the average lobster population is 4.0 ± 0.4% and expected heterozygosity averages 4.6 ± 0.5%. The two European populations both show overall excess homozygosity as in *H. americanus* populations. Mean difference between observed and expected average heterozygosity, $d = -0.67\%$, is highly significant for the ten paired observations ($t = 5.49$, $P < 0.001$).

TABLE II

Allozyme variation at ten polymorphic loci in two populations of the European lobster, Homarus gammarus.

Gene	Population	Sample Size*	Allelic frequencies				Proportions of heterozygotes	
							Observed	Expected**
Acph-1			*98*	*100*				
	Norway	16	0.19	0.81			0.12	0.32
	Irish Sea	36	0.31	0.69			0.28	0.44
Acph-2			*96*	*100*				
	Norway	16	0.93	0.07			0.14	0.14
	Irish Sea	16	1.00	—			—	—
Acph-5			*99*	*100*				
	Norway	16	1.00	—			—	—
	Irish Sea	40	0.58	0.42			0.55	0.50
Est-2			*99*	*100*				
	Norway	32	0.72	0.28			0.31	0.42
	Irish Sea	36	0.53	0.47			0.50	0.51
Me			*98*	*100*	*102*	*104*		
	Norway	14	—	—	0.93	0.07	0.14	0.14
	Irish Sea	38	0.08	0.39	0.53	—	0.58	0.58
Pgi-3			*95*	*100*				
	Norway	60	0.02	0.98			0.03	0.03
	Irish Sea	40	—	1.00			—	—
Pgi-4			*96*	*98*				
	Norway	60	0.07	0.93			0.13	0.13
	Irish Sea	68	0.02	0.98			0.03	0.03
Pgm-1			*100*	*103*				
	Norway	60	—	1.00			—	—
	Irish Sea	46	0.02	0.98			0.04	0.04
Pgm-2			*95*	*100*				
	Norway	16	0.19	0.81			0.38	0.33
	Irish Sea	38	—	1.00			—	—
Tpi			*100*	*107*				
	Norway	60	0.98	0.02			0.03	0.03
	Irish Sea	72	0.97	0.03			0.06	0.06

* Estimated number of genomes sampled (see text).

** Expected genotype proportions calculated by Levene's formulae for small samples (Levene, 1949).

Genetic similarity of Norway and Irish Sea H. gammarus *populations*

The two population samples of *H. gammarus* appear to have different allelic frequencies at several polymorphic loci (Table II). The most substantial divergences are at *Acph-5* (Nei's genetic identity, $I = 0.80$), and at the *Me* locus ($I = 0.79$), the latter a parallel to *Me* differentiation among *H. americanus* populations (Tracey *et al.*, 1975). Sample sizes of the *H. gammarus* populations are too small to permit much confidence in the allelic frequency estimates of Table II, but, on the whole, gene frequencies appear quite similar. Norway and Irish Sea samples are monomorphic for the same allele at an additional twenty-six loci. As a result, the average genetic identity of conspecific *H. gammarus* populations is $\bar{I} = 0.987$. For all conspecific lobster population comparisons $\bar{I} = 0.994 \pm 0.001$ and $\bar{D} = 0.006 \pm 0.001$.

Genetic divergence of Homarus *species*

Identity and genetic distance statistics were computed for all loci studied in all pairwise combinations of European and American lobster populations. The results are summarized under four classes of genetic divergence: (A) gene-enzyme systems monomorphic, or nearly so, for the same electromorph in both species; (B) gene-enzyme systems monomorphic, or nearly so, for different electromorphs; (C) polymorphic systems sharing the same allozymes in both species; and (D) polymorphic systems showing species differences in electrophoretic profile (Table III).

The majority of loci (30/41) show no variability or divergence between species, nine polymorphic loci show a wide range of *I* values, and only two loci show fixed or nearly fixed differences between the species. New alleles found to be specific to *H. gammarus* are *Acph-5*^{99}, *Me*^{98}, and *Me*^{104}, *Pgi-4*^{96}, *Pgm-2*^{95}, and *Tpi*^{107}.

Since both fixed differences are acid phosphatases, one might question whether these mobility differences are due to two loci or to one locus that encodes a

TABLE III

Distribution of gene-enzyme systems according to four classes of similarity in comparisons between European and American lobster populations.

	Number of loci	Range of *I**
A. Loci fixed for same allele *Acph-4, Est-4, Est-5, Est-6, Est-8, Fum-1, Fum-2, Got, G-3pdh, Hk-1, Hk-2, Hk-3, Idh, Mdh-1, Mdh-2, Per, 6-Pgdh, Pgi-1, Pt-1, Pt-2, Pt-3, Pt-4, Pt-5, Pt-6, To-1, To-2, To-3, To-4, To-5, Tr-4.*	30	>0.99
B. Loci fixed for different alleles *Acph-2, Acph-3*	2	0.0 0.08
C. Polymorphic loci sharing alleles *Acph-1, Est-2, Pgi-3, Pgm-1*	4	0.4–1.0
D. Polymorphic loci having different allelic profiles *Acph-5, Me, Pgi-4, Pgm-2, Tpi*	5	0.0–1.0

* Nei's (1972) statistic of genetic identity.

polypeptide subunit common to both enzymes. ACPH-2 is specific to testis and migrates about 4 mm less than an ACPH-3 band specific to green gland tissue. In *H. gammarus,* both of these enzymes migrate 4 or 5 mm less than the *H. americanus* bands, so a single locus change is a reasonable hypothesis. However, one Norway male appeared to be heterozygous, *Acph-2*$^{96/100}$, indicating that this ACPH is dimeric and that the *H. americanus* allozyme occurs in European lobster populations at low frequencies. Since this individual was at the same time homozygous at *Acph-3* for the typical *H. gammarus* allele, the two ACPH's must be encoded by at least two loci.

Nei's (1972) statistics of I and D averaged over all loci and interspecific population comparisons are: $\bar{I} = 0.896 \pm 0.007$ and $\bar{D} = 0.110 \pm 0.007$. Under certain assumptions, $\bar{D}$ estimates the average number of electrophoretically detectable amino acid substitutions per protein since divergence of the two species (Nei, 1972). Thus, eleven such codon substitutions are estimated to have occurred for every 100 proteins.

Progeny studies

A minimum of eight eggs from each ovigerous female was examined for variants of each of the thirteen proteins listed above. Phenotypic variation within progenies was detected only in the Norway sample and only for esterase-2, triosephosphate isomerase, and phosphoglucose isomerases-3 and -4. Only TPI and PGI-3 F_1 phenotypes conformed to single locus genetic models, as follows.

Progeny from one HGN female showed two TPI phenotypes: the common single-banded phenotype and a three-banded pattern consisting of the common band, a band migrating 7 mm farther, and an intermediate, heavier-staining band (*i.e.,* the classic dimer heterozygote zymogram). The female, herself a triple-banded presumptive *Tpi* heterozygote, hatched homozygous and heterozygous progeny in a ratio of 20: 15, not significantly different than 1 : 1. TPI electrophoretic mobility in *H. gammarus* populations is apparently controlled by a single locus having two alleles, *Tpi*^{100} and *Tpi*^{107}.

Another HGN female proved to be heterozygous at *Pgi-3*$^{95/100}$. Her progeny consisted of *Pgi-3*$^{95/100}$ heterozygotes and *Pgi-3*$^{100/100}$ homozygotes in the ratio 69: 57, not significantly different than 1 : 1. This is in accord with observations of PGI-3 inheritance in *H. americanus* (Hedgecock *et al.,* 1975). However, apparent isozyme formation between PGI-3 and PGI-4 in these *H. gammarus* heterozygous F_1 was the first evidence of interaction between these two enzymes. Progeny with the *Pgi-3*$^{95/100}$ genotypes uniformly exhibited, in addition to the three banded PGI-3 heterozygous pattern and a doubling of the PGI-3 satelite band, a doubling of the lower band in the PGI-4 zone. Such PGI zymograms are observed in fishes that have a duplication of the *Pgi* locus (Avise and Kitto, 1973). For the American lobster PGI patterns this explanation is unsatisfactory since PGI-4 appears to be a monomer (Hedgecock *et al.,* 1975). PGI isozyme structure in lobsters requires further study.

DISCUSSION

As a whole, the genus *Homarus* is characterized by a rather low level of gene-enzyme variation relative to other invertebrates. For both the European and

American species, there are on the average 1.2 alleles per locus, one out of five loci polymorphic per population, and one out of every twenty-five loci heterozygous per individual genome. Average heterozygosity for invertebrates tends to be much higher; Selander and Kaufman (1973) find an average proportion of heterozygous loci per individual of 15.1% for 24 invertebrate species. In more recent investigations of marine invertebrates, however, average heterozygosity does range from 1.1% (*Asterias vulgaris;* Schopf and Murphy, 1973) to 22% (*Tridacna maxima;* Ayala, Hedgecock, Zumwalt and Valentine, 1973).

Certainly, *Homarus* is not atypical when compared with other decapod crustacea. A survey of eleven species representative of most of the major subdivisions of the order Decapoda on the west coast of North America yields an average heterozygosity of about 5.8% (Hedgecock *et al.,* 1976, and unpublished data). In contrast to most other invertebrates that have been studied, decapods tend to be large, mobile, omniverous animals occupying vast geographical ranges. Lower heterozygosity in such animals is thus compatible with the hypothesis that genetic variability is not maintained in species pursuing "fine-grained" adaptive strategies (Levins, 1968; Selander and Kaufman, 1973). Since all decapods studied have similar temperate distributions, the observations are likewise compatible with the notion that homozygosity evolves in environments with temporally (seasonally) fluctuating trophic resources (Valentine, 1976).

Genic heterozygosity in *Homarus* is concentrated at four or five loci in each species; but with the exceptions of ubiquitous *Est-2* and *Acph-1* polymorphisms, the two sets of variable loci differ. Such a distribution of variability over loci is typically observed (Selander, 1976).

Slight, but significant, heterozygote deficiency with respect to Hardy-Weinberg expected proportions is consistently observed. One way such departures may arise is by pooling together in a sample individuals from two or more differentiated demes (Wahlund effect). On a broad geographical scale at least, *Me* allele frequency divergence provides more direct evidence that populations of both species are indeed subdivided.

Divergence between the species of *Homarus* is rather small compared to differences between other congeneric animal species that have been studied. Even among morphologically similar or indistinguishable sibling species genetic distance is substantial [$\bar{D} = 0.581$, in the *Drosophila willistoni* group (Ayala, Tracey, Hedgecock and Richmond, 1974); and $\bar{D} = 0.672$ between the naturally hybridizing sea stars, *Asterias forbesi* and *A. vulgaris* (Schopf and Murphy, 1973)]. However, like *Homarus,* some morphologically distinct species of fishes, rodents and annual plants are characterized by small $\bar{D}$ values, in the range 0.02 to 0.15 (see review of Ayala, 1976).

Thus, species differences in *Homarus* support the notion that structural gene evolution (as measured by electrophoresis) may proceed independently and at a different rate from evolution at more complex phenotypic levels (King and Wilson, 1975; Wilson, Maxson and Sarich, 1974; Wilson, Sarich and Maxson, 1974). Moreover, since rates of protein evolution appear to be proportional to time (*ibid;* Ayala, 1976; Carson, 1976), great similarity in the electrophoretic profiles of European and American lobsters ($\bar{D} = 0.11$) may reflect fairly recent evolutionary divergence. Crude estimates of a divergence time may be calculated either from theoretical equations (Nei, 1972) or from observed rates of protein evolution (for example, Yang, Soulé and Gorman, 1974; Carson, 1976); for *Homarus* these range from 82,000 years by the former method to 2 million years B.P. by the latter and suggest a Pleistocene separation. It is plausible that geographic isolation of American and European lobster populations did occur as the result of Pleistocene glaciation. (Figure 1, CLIMAP Project Members, 1976, illustrates, for example, the compression of temperate zone habitat along the Atlantic margins of Europe and North America at the height of the most recent glaciation, 18,000 B.P.) Since *Homarus* is today absent from the waters around Greenland and Iceland (A. Gardarsson, University of Iceland, personal communication), the two species of lobster have apparently been isolated at least throughout the late Pleistocene and Recent periods. "No choice" laboratory matings have produced viable F_1 hybrids (J. Carlberg and J. Van Olst, California State University, San Diego, personal communication; personal observation). Although more thorough characterization of pre- and postzygotic reproductive isolating mechanisms must be made, gene exchange between the two species appears possible at least under culture conditions. Interspecific hybridization is thus implicated as a potentially important means of introducing genetic variability into lobster broodstock.

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SUMMARY

European lobsters (*Homarus gammarus*) from the Norway coast and from the Irish Sea are examined for electrophoretically detectable genetic variation in seventeen functionally different proteins. Forty-one loci encoding these proteins are homologous with loci studied in a previous survey of eight populations of *H. americanus.*

Progeny hatched from ovigerous Norway females show variation in three enzymes, but Mendelian inheritance is confirmed only for triosephosphate isomerase and for one of the phosphoglucose isomerases. Complex PGI phenotypes are described.

The average amounts of genetic variability in European and American lobster populations appear to be equivalent. More than one allele is detected at 20% of the loci, the average number of alleles detected per locus is 1.2 and the average proportion of loci heterozygous per individual is 4.0%. While much less genically variable than other invertebrates, *Homarus* is not atypical when compared with eleven decapod species that average 5.8% heterozygosity. This is consistent with hypotheses relating genetic variability to adaptive strategy.

At thirty loci *H. gammarus* is monomorphic for the common *H. americanus* allele. Two acid phosphatase systems are fixed or nearly fixed for alternative alleles in the two species while the remaining polymorphic loci show various degrees of interspecific divergence. Unique *H. gammarus* alleles are detected at five loci but only contribute significantly to species differences at the *Acph-5*, *Me*, and *Pgi-4* loci. *Acph-1*, *Est-2*, *Pgi-3*, and *Pgm-1* are polymorphic for the same alleles in both species, but again, with various differences in allelic frequencies. In sum, average genetic identity and average genetic distance are: $\bar{I} = 0.896 \pm 0.007$ and $\bar{D} = 0.110 \pm 0.007$, respectively. Compared to the values for conspecific population comparisons, $\bar{I} = 0.994 \pm 0.001$ and $\bar{D} = 0.006 \pm 0.001$, it is clear that a small but significant amount of genetic divergence separates the European and American lobster.

Based on the premise that protein differences between existing species reflect the amount of time since they shared a common ancestor, it can be speculated that the European and American lobsters were isolated during the Pleistocene. The apparent weakness of reproductive isolating barriers suggests that these populations have evolved allopatrically.

Finally, quantification of species' genetic differences, together with recent successes in interspecific laboratory matings, implicates species hybridization as a potentially important breeding practice in lobster aquaculture.

LITERATURE CITED

AVISE, J. C. AND G. B. KITTO, 1973. Phosphoglucose isomerase gene duplication in the bony fishes: an evolutionary history. *Biochem. Genetics*, **8**: 113–132.

AYALA, F. J., 1976. Genetic differentiation during the speciation process. Pages 1–78 in Th. Dobzhansky, M. K. Hecht, and W. C. Steere, Eds., *Evolutionary biology, Volume 8.* Appleton-Century-Crofts, New York.

AYALA, F. J., D. HEDGECOCK, G. S. ZUMWALT, AND J. W. VALENTINE, 1973. Genetic variation in *Tridacna maxima*, an ecological analog of some unsuccessful evolutionary lineages. *Evolution*, **27**: 177–191.

AYALA, F. J., M. L. TRACEY, D. HEDGECOCK, AND R. C. RICHMOND, 1974. Genetic differentiation during the speciation process in *Drosophila*. *Evolution*, **28**: 576–592.

CARSON, H. L., 1976. Inference of the time of origin of some *Drosophila* species. *Nature*, **259**: 395–396.

CLIMAP PROJECT MEMBERS, 1976. The surface of the ice-age earth. *Science*, **191**: 1131–1137.

HEDGECOCK, D., K. NELSON, R. A. SHLESER AND M. L. TRACEY, 1975. Biochemical genetics of lobsters (*Homarus*). II. Inheritance of allozymes in *H. americanus*. *J. Hered.*, **66**: 114–118.

HEDGECOCK, D., R. A. SHLESER, AND K. NELSON, 1976. Applications of biochemical genetics to aquaculture. Proceedings of the XIII Pacific Science Congress. *J. Fish. Res. Board Can.*, **33**: 1108–1119.

KING, M. C. AND A. C. WILSON, 1975. Evolution at two levels in humans and chimpanzees. *Science*, **188**: 107–116.

LEVENE, H., 1949. On a matching problem arising in genetics. *Ann. Math. Stat.*, **20**: 91–93.

LEVINS, R., 1968. *Evolution in changing environments.* Princeton University Press. Princeton, New Jersey, 120 pp.

NEI, M., 1972. Genetic distance between populations. *Amer. Natur.*, **106**: 283–292.

NEI, M. AND A. K. ROYCHOUDHURY, 1974. Sampling variances of heterozygosity and genetic distance. *Genetics*, **76**: 379–390.

SELANDER, R. K., 1976. Genic variation in natural populations. Pages 21–45 in F. J. Ayala, Ed., *Molecular evolution.* Sinauer, Sunderland, Massachusetts.

SELANDER, R. K. AND D. W. KAUFMAN, 1973. Genic variability and strategies of adaptation in animals. *Proc. Nat. Acad. Sci. U.S.A.*, **70**: 1875–1877.

Schopf, T. J. M. and L. S. Murphy, 1973. Protein polymorphism of the hybridizing sea stars *Asterias forbesi* and *Asterias vulgaris* and implications for their evolution. *Biol. Bull.*, 145: 589–597.

Tracey, M. L., K. Nelson, D. Hedgecock, R. A. Shleser and M. L. Pressick, 1975. Biochemical genetics of lobsters (*Homarus*): genetic variation and the structure of American lobster populations. *J. Fish. Res. Board Can.*, **32**: 2091–2101.

Valentine, J. W., 1976. Genetic strategies of adaptation. Pages 78–94 in F. J. Ayala, Ed., *Molecular evolution*. Sinauer, Sunderland, Massachusetts.

Wilson, A. C., L. R. Maxson, and V. M. Sarich, 1974. Two types of molecular evolution. Evidence from studies of interspecific hybridization. *Proc. Nat. Acad. Sci. U.S.A.*, **71**: 2843–2847.

Wilson, A. C. ,V. M. Sarich, and L. R. Maxson, 1974. The importance of gene rearrangement in evolution: evidence from studies on rates of chromosomal, protein and anatomical evolution. *Proc. Nat. Acad. Sci. U.S.A.*, **71**: 3028–3030.

Yang, S. Y., M. Soulé, and G. C. Gorman, 1974. *Anolis* lizards of the eastern Caribbean: a case study in evolution. I. Genetic relationships, phylogeny, and colonization sequence of the *roquet* group. *Syst. Zool.*, **23**: 387–399.

Reference: *Biol. Bull.*, **152**: 51-63. (February, 1977)

DEVELOPMENT OF *AMPHIOPLUS ABDITUS* (VERRILL) (ECHINODERMATA: OPHIUROIDEA): I. LARVAL BIOLOGY [1], [2]

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A novel pattern of ophiuroid development which involves the rapid direct development of an abbreviated pluteus-like embryo within a demersal fertilization membrane occurs in *Amphioplus abditus,* a burrowing shallow-water amphiurid of the east coast of the United States (Hendler, 1973). Development of *A. abditus* within a fertilization membrane, although resembling that found in certain sipunculids (Rice, 1967), polychaetes (Thorson, 1946; Davis, 1968), archiannelids (Ax, 1966), and holothuroids (Edwards, 1909; Ohshima, 1925), is previously unknown in ophiuroids and therefore warrents detailed description.

MATERIALS AND METHODS

Specimens of *A. abditus* were collected from 1–3 m depths off Noank, Connecticut, at the northeastern end of Long Island Sound. Juveniles were reared during June and July, 1971 and 1972, from females which spawned naturally in the laboratory. Eggs were removed from fingerbowls with spawning females, transferred to small containers of millipore-filtered sea water, and immediately fertilized with freshly shed spermatozoa or sperm from stripped testes. Culture dishes routinely were held between 21° and 22° C in a running seawater bath. To test temperature tolerance, a culture initiated July 14, 1971, was held at 16° C. Culture water was replaced with fresh millipore-filtered sea water every other day.

Viable embryos identical to those reared in the laboratory were collected in the field from superficial sediment using SCUBA and the "Clarksucker", a small suction sampler (Clark, 1971). Observations were made on live specimens, using phase contrast; measurements were made with an ocular micrometer; and drawings prepared with the aid of a camera lucida.

Brine was prepared by freezing sea water, and dilutions of brine or sea water were used to test larval salinity tolerance. Seawater concentration required for successful hatching was gauged by subjecting mature ova, triangular embryos, circular ophiuroid disc, star-shaped ophiuroid disc stages, and newly hatched juveniles grown in 33‰ salinity sea water to 5, 10, 15, 20, 25, 30, 35, and 40‰ salinity. (The above developmental stages are described in Results). Three replicate tests were made of each of the combinations of eight salinities and four developmental stages and each replicate employed eggs from a single female. The subcultures were maintained in 50 ml beakers with 20 ml of test solution and 50–100 embryos (greater densities resulted in retarded growth). Every one to two days the solution in these cultures was changed. Numbers of embryos surviving or hatched were determined at 12- to 24-hour intervals by examination with a dissecting microscope, and these cultures were maintained up to ten days in order to test for survival.

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[1] University of Connecticut Marine Research Laboratory Contribution No. 105.

[2] Parts of this paper were submitted in partial fulfillment of the requirements for a Ph.D. at the University of Connecticut.

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RESULTS

Development of Amphioplus abditus

Spawning. It usually is impossible to rear ophiuroid eggs stripped from ovaries. Forced spawning by exposing ripe individuals to artificial illumination after sunset (Grave, 1900; Mortensen, 1921, 1937; Olsen, 1942; Fenaux, 1963) or after dark-adaptation (Stancyk, 1973) is resorted to most frequently. On seven occasions female specimens of *A. abditus*, collected in the afternoon and held under constant illumination in unaerated sea water, spawned in the laboratory between 1800 and 0100 hours. This spawning after dark may have depended upon the time of collection, rather than an intrinsic nocturnal behavior pattern. Males held in a container with females generally shed spermatozoa after the females spawned. *Amphioplus abditus* was never observed while spawning during day or night dives, nor in laboratory tanks with sediment and running or standing sea water.

In fingerbowls with only seawater, their spawning posture is similar to that described for the burrowing species *Amphiura filiformis* (Mortensen, 1920; Woodley, 1975), *Amphiura chiajei* (Mortensen, 1920), and *Amphiodia barbarae* (MacGinitie and MacGinitie, 1949), an attitude described also in epibenthic species such as *Ophioderma brevispinum* (Grave, 1916), *Ophiopholis aculeata, Ophiura albida, Ophiura texturata, Ophiocomina nigra, Ophiothrix fragilis* (Olsen, 1942). The disc is raised several centimeters above the substrate by the proximal part of the arms, with the distal length of the arms radiating and flattened against the substrate to form a base. In their natural habitat this behavior would probably raise the disc above the sediment. The disc is never autotomized during spawning, ruling out the possibility that spawning results in natural loss of the disc and regeneration in amphiurid brittlestars (Clark, 1970).

During spawning, movement of the arms and oral skeleton produces waves of contraction superimposed on the rhythmical and strenuous contraction/relaxation of the muscles of the disc. These spawning labors are intermittent before, and continuous during shedding of gametes. Gametes are emitted simultaneously from all five bursae. The eggs are shed in loose streams and, although the proximal tube-feet move rapidly and might serve to carry gametes out of the ophiuroid's burrow, those shed in the laboratory fell directly to the substrate without contacting the tube-feet. Ova not immediately fertilized develop abnormally. Spermatozoa, also shed in strings, disperse rapidly and remain capable of movement for at least three hours.

Eggs are shed for about twenty minutes. Since all eggs are not shed in one spawning, and individuals are "spent" by August, there must be two or more spawnings during the two-month breeding period. Sperm is released spasmodically and the shedding time of males generally exceeds that of females, possibly an adaptation to facilitate external fertilization, ensuring that sperm will be available throughout the period of egg release.

Egg maturation. Eggs which spawn naturally lack the germinal vesicles present in oocytes within the ovaries. One female (fixed during spawning, then embedded, and sectioned) had some oocytes with meiotic spindles within its ovaries. The oocytes were apparently shed during or shortly after the first meiotic division, as no polar bodies were seen until after fertilization. This may be a widespread phenomenon among ophiuroids, since polar bodies have been noted after fertilization for other species (Nachtrieb, 1885; Olsen, 1942; Fell, 1946), although observations in this regard for *Gorgonocephalus caryi* are equivocal (Patent, 1969).

The dark green-gray eggs are homolecithal. Newly-laid eggs are spherical or slightly ovoid and surrounded by extremely thin membrane(s). Within one minute after fertilization the outermost layer of the egg first becomes clear and then granular. Next, the fertilization membrane begins to expand away from the egg. As the perivitelline space enlarges, the surface of the egg wrinkles and its shape changes (Figure 1a). Simultaneously, the surface of the egg is covered with blebs which round off and then disassociate from the surface (Figure 1a). As they migrate and disappear, they seem to produce strands of material in the perivitelline space and leave stellar patterns on the fertilization membrane. These processes occur within 10 minutes, and within 30 minutes the egg again becomes spherical. It is usually eccentric within the expanding fertilization membrane and may be supported by cytoplastic elements from the cortical layer. Changes in the length of the egg (Figure 1a) indicate the extent of deformation during wrinkling. The length of major axis of the egg in microns (mean ± standard deviation, and number of measurements) changed from 132.12 ± 2.24 (10) for unfertilized ova; to 148.92 ± 1.41 (6) for wrinkled ova; and to 128.12 ± 2.00 (10) for ova after membrane elevation. The decrease in the diameter after fertilization was found to be significant using the Student-Newman-Keuls test.

Shortly after the ovum rounds off, the diameter of the fertilization membrane reaches a maximum of 417.88 ± 21.88 μ (s.d.). This leaves a perivitelline space in *A. abditus* of almost 150 μ, as compared with 10 μ in most other ophiuroids (Grave, 1898; Olsen, 1942; Patent, 1970) or 50 μ, at most (Narasimhamurti, 1933; Guille, 1964).

The expanded fertilization membrane of *A. abditus* is adhesive. Stickiness must be a property only of the expanded membrane since the newly shed eggs surrounded by a vitelline membrane are not adhesive. After the fertilization membrane is formed, and throughout development, the eggs adhere to the substrate. They stick to glass culture containers from which water has been decanted, and, if dislodged, they can settle and readhere.

The change in shape of the ovum of *A. abditus* following fertilization corresponds to the "angular phase" of *Ophiopholis aculeata* (Olsen, 1942) and may be part of a germinal localization process. However, the breakdown of the cortical layer, blebbing, wrinkling, and production of perivitelline structures are more exaggerated in *A. abditus* than in other echinoderms (Endo, 1961). The extremely expansive fertilization membrane may engage an unusual amount of material from the cortical layer since the mean egg diameter decreases 4 μ after wrinkling.

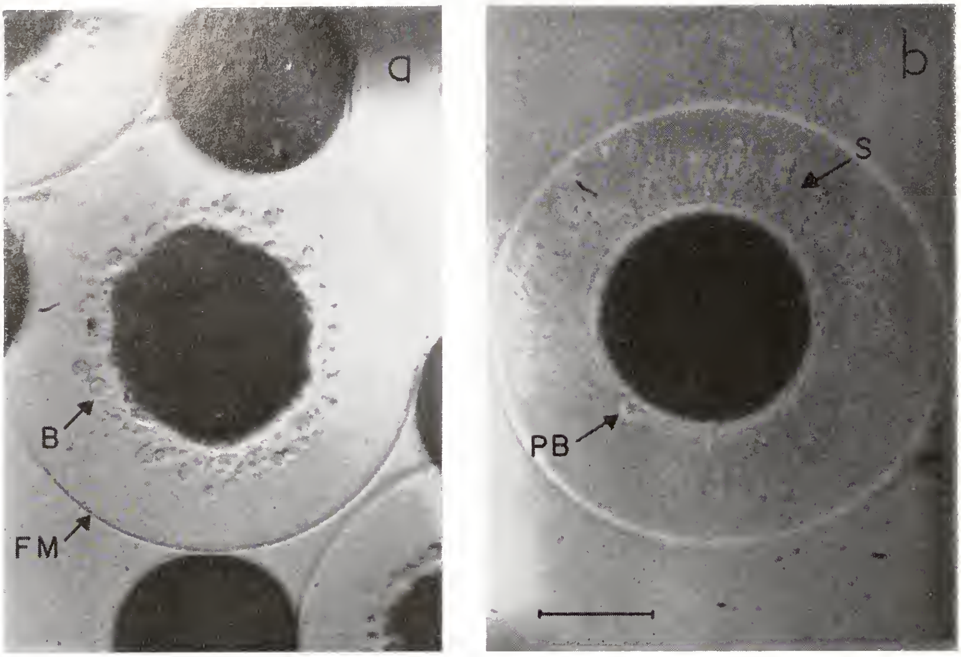

FIGURE 1. Ova of *Amphioplus* after fertilization: a shows an ovum at 16 minutes after fertilization, having irregular, nonspherical proportions, and blebs migrating toward the fertilization membrane; and b, an ovum at one hour, with nearly spherical shape and having strands of material in the perivitelline space and a newly extruded polar body. Scale line is 0.1 mm. Abbreviations are: B, blebs; FM, fertilization membrane; S, perivitelline strands; and PB, polar body.

suggesting loss of a 2 μ layer which might be converted to perivitelline material or the fertilization membrane.

Depending on temperature of the medium, the first polar body may be seen at 30 minutes and the remainder appear by one hour (Figure 1b). The polar bodies are extruded slowly, producing a bulge at the surface of the egg. They are found in a group on the surface of the egg and remain recognizable through the first 3 or 4 cleavages.

Cleavage, blastulation and gastrulation. The first segmentation occurs within 2 hours and the second follows in about 30 minutes, perpendicular to the first. Eight-cell and sixteen-cell stages are reached about 3 and 4 hours after fertilization, respectively. Successive divisions, equal and holoblastic, occur at approximately hourly intervals, producing a nonciliated blastula in 7–10 hours. A hyaline layer appears to invest each cell and does not seal the blastopore during gastrulation.

Gastrulation occurs within 9–12 hours, apparently by invagination, and the embryo afterward assumes a very blunt triangular shape with a small, rounded blastopore at the broad end. The blastopore broadens and widens as gastrulation continues and the embryo takes the shape of an arrowhead with the acute anterior end appreciably darker (denser) than the posterior.

Triangular embryo. By 16 hours after fertilization the embryo reaches a length of 0.23 mm and is entirely ciliated. Embryos can move within the fertilization membrane at this time. In abnormal cases where the fertilization membrane is lacking, embryos are propelled across the bottom of the culture vessel by their cilia, but ordinarily, ciliary movement must serve only to circulate fluid within the fertilization membrane.

There are four important concurrent transformations between 18 and 24 hours. As the body reaches 0.28 mm, the posterior corners of the embryo protrude as rudimentary posterolateral arms (Figure 2a). The edges on either side of the blastopore, between the posterolateral protrusions, thicken and the blastopore is obliterated. Triradiate spicules, rudiments of the larval skeleton, appear near the posterolateral protrusions. Toward the apex of the embryo, the anterior end, the hydrocoel becomes visible as a curved, five-scalloped structure.

The embryo assumes a recognizably new and distinct form after 30 hours of development (Figure 2b). It has reached its maximum length, but the posterolateral arms extend almost perpendicular to the body so that the width exceeds

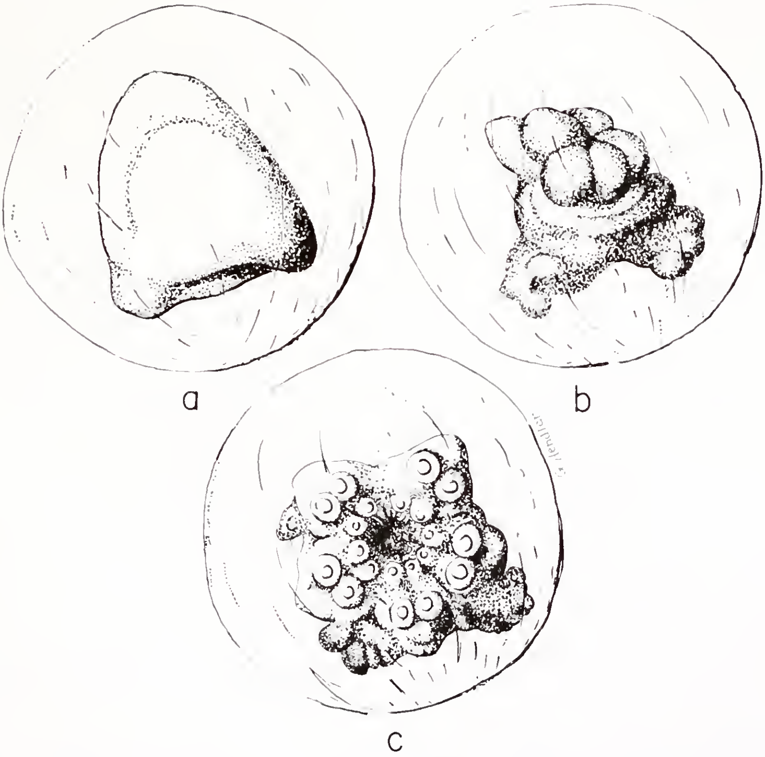

FIGURE 2. Major embryonic stages of *Amphioplus*. Neither perivitelline structures nor ciliation are shown, but fertilization membranes are illustrated. Embryos are each approximately 0.3 mm long, and fertilization membranes about 0.4 mm diameter. Drawing a shows an advanced triangular embryo with rudimentary posterolateral arms and an elevated area surrounding the hydrocoel, 24 hours; b, an early circular disc stage with emerging opiuroid rudiment and plamate embryonic arms, 34 hours; and c, star disc stage with reduced embryonic structures and a prominent ophiuroid rudiment having podia and mouth.

the length. The anterior ventral surface of the body bulges and beneath the protrusion there is a hydrocoel with five large lobes. The larval skeleton, extending to the base of the arms, has become tetraradiate, elongate, and bilaterally symmetrical. Cilia still cover the entire body but are most noticeable on the arms.

Circular ophiuroid disc. Between 34 and 38 hours the ophiuroid rudiment emerges as a circular disc on the embryo (Figure 2b). An indentation, the presumptive mouth, appears at the center of the disc and five lobes form along the edge of the disc. The tips of the embryonic arms become indented, giving them a palmate appearance, and the only prominent ciliation is on the distal edges of the arms.

The five radii of the disc clearly indicate the rudimentary ophiuroid arms by 40 hours. Shortly afterward, 20 tube-foot rudiments can be distinguished, two on each side of each presumptive arm. The distal pairs are larger, and appear to form earlier, than the proximal. This indicates that the ends of the hydrocoel have already fused below the enlarging ophiuroid mouth. At this stage the tips of the larval skeleton bifurcate and the ophiuroid skeleton consists of six triradiate primary plates, five tetraradiate terminal plates, and ten to twelve tiny rudiments of the oral skeletal elements. The shape of the ophiuroid body becomes even clearer by 45 hours as the arm tips sharpen and the tube-feet become erect papillae. The embryonic body becomes less prominent, with the embryonic arms scarcely wider than the ophiuroid body. At the same time, the larval skeleton continues to grow, becoming more clearly visible as yolk reserves are depleted, and the embryonic arms become transparent.

Star-shaped ophiuroid disc. As the ophiuroid disc rudiment takes a pentagonal shape, the mouth, tube-feet, and arms enlarge (Figure 2c). The anterior tip of the embryonic body and the embryonic arms, still paddle-shaped, become smaller and increasingly transparent, while the larval skeleton becomes more complex. Cilia, sparse but still active on the larval armtips by 50 hours, continue to disappear from the body. The tube-feet, the first part of the ophiuroid rudiment to move, begin to wave at about 55 hours. The more proximal tube-feet are shifted toward the enlarging oral cavity and increased transparency in the buccal area indicates the stomach is enlarging. By this time ophiuroid skeletal elements have advanced in size by branching, but the larval skeleton is noticeably simpler by 60 hours and the anterior tip of the larval body takes on a pinched papillate shape owing to resorption. Within another few hours the ophiuroid body begins to move and the increased coordination of the musculature of the body matches a concomitant increase in the complexity of the ophiuroid skeleton.

By 70 hours of development the triangular gastrula shape is obscured by the pentagonal ophiuroid form. The apex of the embryo is insignificant; embryonic arms are small, clear, and devoid of ciliation; and the larval skeleton diminishes. The ophiuroid skeleton continues to branch and anastomose, forming plates of solid outline, and the tube-feet lengthen. Between 75 and 85 hours, the total resorption of embryonic structures and the development of the juvenile ophiuroid occur *pari passu.* During this period the ophiuroid is increasingly active. It moves about within the fertilization membrane, often buckling the membrane as it retracts its tube-feet. The terminal plates of the arm also distort the shape of the membrane and sometimes scratch it.

Hatching and juveniles. Hatching, the escape from the fertilization membrane, may begin within 90–95 hours and in a single culture may continue for several days Since the young are at the same stage of development, the prolonged period of hatching must be a function of the strength of the fertilization membrane or of the juvenile. Emergence is through a slit in the fertilization membrane, probably produced by the pressure of the terminal plates. During emergence the tube-feet are used to push against the membrane and pull on the substratum. The fertilization membrane is discarded and not used as food.

Newly hatched juveniles with disc diameters of 0.3 mm (radius from mouth to tip of arm = 0.21 mm) move about continually. They walk on the distal podia (second buccal tube-feet of the adult), often rearing up, raising the entire body on a pair of podia and sometimes using the adoral shield spines for leverage. In fingerbowls they usually concentrate on the bottom edge but sometimes climb or float upside-down under the surface film. Offered fine sediment, they alternately move across the surface and burrow.

The podia are adapted in shape and secretory ability for locomotion. Distinct patches on the column and the entire tip of the podia, as well as the epidermis bordering the edge of the disc, stain metachromatically with toluidine blue, indicating localized mucous production. In addition, the tips of these ambulatory podia (not the terminal or first buccal tube-feet) possess two plus three opposing papillae. These are lacking from podia of adult specimens and they may be a modification to increase surface area of the tips of the juvenile's tube-feet for increased traction. Detrital material adheres primarily to the tube-feet of juveniles, whereas in adults the spines are more important than tube-feet as mucous-secreting structures.

Juveniles lose the green coloration of the egg and embryo as the skeletal plates enlarge. Within two weeks, detrital material is ingested giving the stomach a deep-brown color and causing the disc to bulge upward.

Growth in the laboratory is very slow. Juveniles hatched in July, and maintained at 15° C from the end of August, produced one arm-segment in 2 months, three at about 3 months, and four by 8 months. Although conditions for growth were probably not optimal, the temperature was higher than that in the field over the winter so that accelerated growth might have been expected. The growth rate differs from Mortensen's (1920) observation that *Amphiura filiformis* reaches the 4 arm-segment stage in 4 months in both laboratory and field. Even the 4 to 8 arm-segment stages of *A. abditus* have tube-feet with papillate tips and move on the tube-feet rather than by movement of the arms. They progress with one arm leading and two trailing, moving alternately under or on top of the sediment. Evidently the adult habit of maintaining a burrow is not adopted until a larger size is attained.

Embryonic tolerance

Temperature. Development was faster in cultures raised at 21° C than at 16° C. Cultures at 16° C developed only to a point where the embryonic body was nearly resorbed; they reached this stage (normally found by 84 hours) at 156 hours, failed to hatch, and died at nine days after fertilization, probably due to exhaustion of food reserves. Q_{10} for rate of development calculated for different

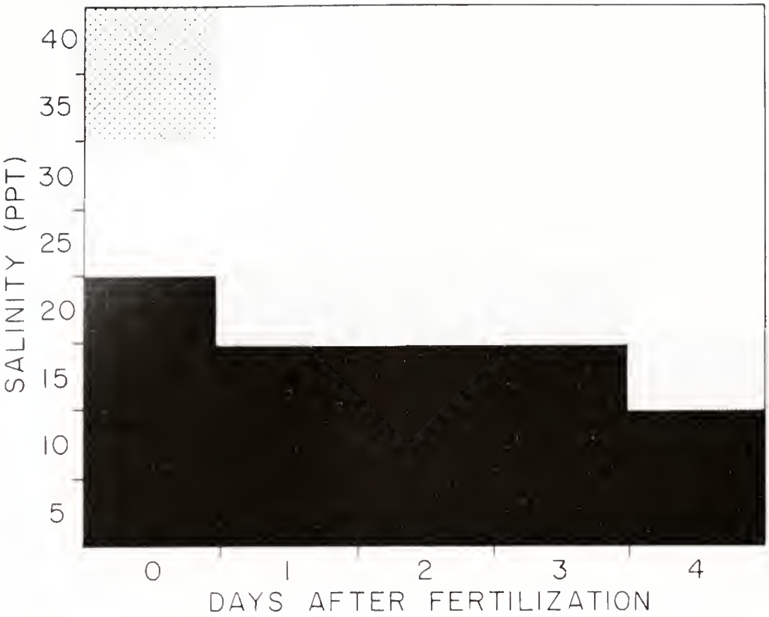

FIGURE 3. Success or failure of hatching for five developmental stages raised at eight salinities. The stages transferred to test salinities were: mature ova, 1.5 hours; triangular disc, 12 hours; circular ophiuroid disc, 12 hours; star-shaped ophiuroid disc, 36 hours; and newly hatched juveniles, 96 hours. Black represents 100% mortality; stippled, swelling of tissues but all replicates with some hatching success; white, all replicates with extensive hatching success; cross-hatched, two of three replicate cultures with 100% mortality.

stages from gastrulation to movement of podia decreased from 5.7 to 2.3. This indicates that development is temperature sensitive and especially so during early stages of morphogenesis. The failure of cultures to develop at 16° C while ambient field temperatures at the same time were 18° C indicates that small temperature changes may have a critical role in the survival of the species. The temperature-limited stage of the life history is in the embryo, since the adult, in the course of the year, survives 0–25° C and tolerates refrigeration in the laboratory during the summer.

Salinity. Tests of salinity tolerance of *Amphioplus* embryos revealed optimal survival at 25–30‰ salinity sea water. The mortality of embryos was elevated in solutions more concentrated or dilute, with the highest mortality in the most hypotonic solutions tested. The results of these tests are shown in Figure 3. For example, reading the axis for 1-day (12-hr) embryos: dilutions of 5–15‰ were lethal (black band); 20‰ resulted in swollen embryos as a result of osmotic stress, but these cultures hatched; and salinities of 25–40‰ gave normal development and hatching.

In general, salinities of at least 20 and preferably 25–40‰ are necessary for survival. In stages less than one day old, the tolerance range is restricted to 25–30‰ and two of three replicate cultures did not survive 35–40‰. On the other hand, after hatching, tolerance to osmotic stress expands to a range of

15–40‰. This pattern of broadening tolerance is found in other organisms (Kinne, 1964).

Marginally hypotonic sea water caused swelling of tissues within 12 hours. Mortality was greater in cultures at marginally low salinities than in isotonic cultures. In extremely hypotonic solutions cells blanched and disintegrated while fertilization membranes often expanded and sometimes ruptured. At 15‰ the cells of early stages sometimes expanded, but usually separated and then dissolved. At the same salinity, the development of advanced stages stopped and superficial structures sometimes swelled to fill the entire fertilization membrane before the embryo disintegrated.

Mature *A. abditus* transferred from the field to bowls with sea water in different concentrations showed a tolerance range similar to that shown by the juveniles raised in the laboratory, indicating that salinity is equally limiting to all but the earliest stages of development. For adults, salinities of 0–5‰ and 50–75‰ were immediately lethal. Those in 10–15‰ showed irritability for a day before dying but only salinities of 20–40‰ were tolerated for the 36-hour test period.

DISCUSSION

Amphioplus abditus has a superficially orthodox morphogenesis and passes through a reduced pluteus-like stage with vestigial embryonic arms, ciliation, and skeleton. It resembles free-swimming ophioplutei both in the median, ventral origin of the stomodeum and closure of the blastopore, although it lacks a functional embryonic anus and the genesis of the coelom is not fully understood.

Modifications in morphogenesis and the elaboration of a formidable fertilization membrane are involved in the rapid, direct, and dermersal development of *A. abditus* within a fertilization membrane. The chronology of development in free-swimming ophiopluteus larvae suggests that completion of the larval body generally takes one to two weeks while complete metamorphosis takes about a month (Hendler, 1975). Thus, abbreviated development in *A. abditus* must result from accelerated morphogenesis of the ophiuroid rudiment *as well as* the virtual absence of a complex larval body. If only the latter were important, complete development would take two weeks rather than one. This dispatch depends on the economy of direct development with a supply of yolk that eliminates the necessity of feeding, nutrient transfer, and the maintenance metabolism of a complex, active larval body.

The important attributes of the fertilization membrane: its size, strength, and adhesiveness are initimately associated with the mode of direct development of *A. abditus.* The first two properties are prerequisites for direct development *per se.* Fertilization membranes of most ophiuroids enclose minimal perivitelline space and are presumably weakened or dissolved by a hatching enzyme as in echinoids (Kumé and Dan, 1968) and discarded by the ciliated, motile larva. There are several possible mechanisms that restrict *A. abditus* to the fertilization membrane: first, the size of the envelope relative to the embryo and the perivitelline material produced by the cortical bodies prevent mechanical breakage; and secondly, the embryo may lack a hatching enzyme or the membrane may be resistant to such an enzyme. The membrane is resilient, not easily ruptured without injuring the embryo. Naturally, size and strength of the membrane and direct development protect the embryo from physical factors and injury by microorganisms. The fact that the fertilization membrane expands to 0.4 mm must discourage predation by meiobenthic fauna that could destroy an unprotected 0.15 mm zygote. Density of the egg and adhesiveness of the fertilization membrane are responsible for demersal development, because they result in sinking of the egg and restrain its movement along the bottom.

In the field, developing embryos were occasionally recovered from containers set about 0.5 m off the bottom to collect settling plankters. Embryos in various stages of development were recovered from the surface of the sediment, but were never taken in the plankton sampled by pump or townet. This indicates that the eggs, embryos, and possibly juveniles, though not planktonic, may be dispersed for short distances by bottom disturbances or currents.

The salinity tolerance of larval and adult specimens of *A. abditus* (roughly 20–40‰) is as broad as many estuarine organisms and approaches the lower limit for echinoderms (Kinne, 1964; Binyon, 1966). Though echinoderm larvae are notoriously less tolerant to low salinity than larvae of lamellibranchs, gastropods, and polychaetes, the rapid demersal development of *A. abditus* suits it for estuarine conditions (Thorson, 1946). For the Mystic River estuary where *A. abditus* was collected, the salinity in vertical transect ranges from 3–30‰ (Pearcy and Richards, 1962). *Amphioplus abditus* cannot tolerate this range and survives where it can avoid salinity stress. The eggs adhere to the bottom where the estuarine salinity is highest and temperature and salinity are least variable; restricted dispersal keeps embryos in a suitable milieu, and rapid development reduces exposure to temperature and salinity fluctuations. Accurate temperature and salinity tolerances have been measured for the larvae of only one other echinoderm, the asteroid *Acanthaster planci*. Lucas (1973) found that *A. planci* had a narrow thermal tolerance but a higher optimum temperature than *A. abditus*. Interestingly, *A. abditus* develops at salinities 20‰ or greater, while *A. planci* required at least 26‰. These differences are not unexpected considering the contrasting environments of the two species: temperate lower estuary and tropical coral reef.

Larvae of estuarine organisms are commonly found to have adaptations to limit their dispersal and evidence is accumulating that demersal development is sometimes adopted (Carriker, 1967; Mileikovsky, 1971; Stancyk, 1973). *Thyone briareus*, a holothuroid commonly sympatric with *A. abditus*, has an abbreviated development within the fertilization membrane (Ohshima, 1925), and it is expected that rapid demersal development within the fertilization membrane will be found for other echinoderms in waters of low salinity.

Direct development in the fertilization membrane may have been found, but not recognized as such, in other ophiuroid species. Nachtrieb (1885) described but did not figure the development of *Ophiophragmus wurdemani*, a shallow-water species from North Carolina and Florida, whose development seems strikingly similar to *A. abditus*. Nachtrieb indicates that the larvae hatch within several days but does not mention a fertilization membrane. Kirk (1916) and Fell (1941) describe an embryo for an unknown ophiuroid species from New Zealand which develops within a "perfectly transparent, thin but extremely tough chitinous envelope . . . deposited in irregular clusters of from 10 to 100 or more" (Kirk, 1916, p. 383). Whether this envelope is a fertilization membrane is yet unknown.

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SUMMARY

1. *Amphioplus abditus*, a burrowing, shallow-water amphiurid brittlestar of the eastern coast of the United States, has a nonplanktonic development within an adhesive, demersal fertilization membrane.

2. The embryo is a ciliated pluteus-like form with a transient blastopore, and vestigial larval arms and skeleton.

3. Development, which takes place on the surface of the sediment, is completed within four days at usual spawning temperature and the slow-growing post-larva, about 0.3 mm disc diameter, is active and capable of burrowing and feeding.

4. Salinity of at least 20–25‰ is necessary for embryonic development and adult survival, but temperature appears to be more critical for embryos than adults and may be an important limiting factor for the geographical range of the species.

5. Rapid, demersal, abbreviated development is an adaptation to a fluctuating, low-salinity environment as it restricts the embryo to the sediment-water interface where salinity is maximal and temperature and salinity are relatively constant. This mode of development has the advantages of direct development but permits limited dispersal capability.

6. The rapid rate of development is attributed to *both* accelerated morphogenesis and the virtual absence of a complex larval body.

LITERATURE CITED

AX, P., 1966. Die Bedeutung der interstitiellen Sandfauna für allgemeine Probleme der Systematick, Okologie, und Biologie. *Veroff. Inst. Meeresforsch. Bremerhaven, Sonderband*, **2**: 15–65.

BINYON, J., 1966. Salinity tolerance and ionic regulation. Pages 359–377 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. Interscience, New York.

CARRIKER, M. R., 1967. Ecology of estuarine benthic invertebrates: a perspective. Pages 442–487 in G. H. Lauf, Ed., *Estuaries*. Amer. Assoc. Advancement Science, No. 83, Washington, D.C.

CLARK, A. M., 1970. Notes on the family Amphiuridae (Ophiuroidea). *Bull. Brit. Mus. (Natur. Hist.) Zool.*, **19**: 1–81.

CLARK, K. B., 1971. The construction of a collecting device for small aquatic organisms and a method for weighing of small invertebrates. *Veliger*, **13**: 364–367.

Davis, C. C., 1968. Mechanism of hatching in aquatic invertebrate eggs. *Oceanogr. Mar. Biol. Annu. Rev.*, **6**: 325–376.

Edwards, C. L., 1909. The development of *Holothuria floridana* Pourtales with especial reference to the ambulacral appendages. *J. Morphol.*, **20**: 211–230.

Endo, Y., 1961. Changes in the cortical layer of sea urchin eggs at fertilization as studied with the electron microscope. I. *Clypeaster japonicus*. *Exp. Cell Res.*, **25**: 283–297.

Fell, H. B., 1941. The direct development of a New Zealand ophiuroid. *Quart. J. Microsc. Sci.*, **82**: 343–377.

Fell, H. B., 1946. Embryology of the viviparous ophiuroid *Amphipholis squamata* Delle Chiaje. *Trans. Roy. Soc. N.Z.*, **75**: 419–464.

Fenaux, L., 1963. Note preliminaire sur le développement larvaire de *Amphiura chiajei* (Forbes). *Vie Mileu*, **14**: 41–46.

Grave, C., 1898. Embryology of *Ophiocoma* Agassiz. *Johns Hopkins Univ. Circ.*, **18**: 6–7.

Grave, C., 1900. *Ophiura brevispina*. *Mem. Biol. Lab. Johns Hopkins Univ.*, **4**: 79–100.

Grave, C., 1916. *Ophiura brevispina*. II. An embryological contribution and a study of the effect of the yolk substance upon development and developmental processes. *J. Morphol.*, **27**: 413–453.

Guille, A., 1964. Contribution a l'etude de la systematique et de l'ecologie de'*Ophiothrix quinquemaculata* d. Ch. *Vie Mileu*, **15**: 243–301.

Hendler, G., 1973. Northwest Atlantic amphiurid brittlestars, *Amphioplus abditus* (Verrill), *Amphioplus macilentus* (Verrill), and *Amphioplus sepultus* n. sp. (Ophiuroidea: Echinodermata): systematics, zoogeography, annual periodicities, and larval adaptations. *Ph.D. thesis, University of Connecticut, Storrs*, 255 pp. (*Diss. Abstr.*, **34**: 3106-B; order no. 74-00007.)

Hendler, G., 1975. Adaptational significance of the patterns of ophiuroid development. *Amer. Zool.*, **15**: 691–715.

Kinne, O., 1964. The effects of temperature and salinity on marine and brackish water animals. II. Salinity and temperature combinations. *Oceanogr. Mar. Biol. Annu. Rev.*, **2**: 281–339.

Kirk, H. B., 1916. On the much-abbreviated development of a sand-star (*Ophionereis schayeri?*).—Preliminary note. *Trans. N.Z. Inst.*, **48**: 383–384.

Kumé, M., and K. Dan, 1968. *Invertebrate embryology.* NOLIT Pub., Belgrade, Yugoslovia, 605 pp.

Lucas, J. S., 1973. Reproduction and larval biology of *Acanthaster planci* (L.) in Great Barrier Reef waters. *Micronesica*, **9**: 197–204.

MacGinitie, G. E., and N. MacGinitie, 1949. *Natural history of marine animals.* McGraw-Hill, New York, 473 pp.

Mileikovsky, S. A., 1971. Types of larval development in marine bottom invertebrates, their distribution and ecological significance: a re-evaluation. *Mar. Biol.*, **10**: 193–213.

Mortensen, T., 1913. Die Echinodermenlarven der Deutschen Südpolar-Expedition 1901–1903. *Südpol.-Exped.*, **14**: 69–111.

Mortensen, T., 1920. Notes on the development and the larval forms of some Scandinavian Echinoderms. *Vidensk. Medd. Naturhist. Foren Kjobenhavn*, **71**: 134–160.

Mortensen, T., 1921. *Studies of the development and larval forms of echinoderms.* G. E. C. Gad, Copenhagen, 261 pp.

Mortensen, T., 1937. Contributions to the study of the development and larval forms of echinoderms. III. *Dan. Kgl. Danske Vidensk. Selsk. Skrifter, Naturv. Math. Afd.*, **9**: 1–65.

Nachtrieb, H. F., 1885. Preliminary notes on the echinoderms of Beaufort. *Johns Hopkins Univ. Circ.*, **4**: 67–68.

Narasimhamurti, N., 1933. The development of *Ophiocomina nigra*. *Quart. J. Microsc. Sci.*, **76**: 63–88.

Olsen, H., 1942. The development of the brittle-star *Ophiopholis aculeata* (O. Fr. Müller), with a short report on the outer hyaline layer. *Bergens Mus. Arb. Naturvid.*, **1942**: 1–107.

Ohshima, H., 1925. Notes on the development of the sea-cucumber *Thyone briareus*. *Science*, **61**: 420–422.

PATENT, D. H., 1969. The reproductive cycle of *Gorgonocephalus caryi* (Echinodermata; Ophiuroidea). *Biol. Bull.,* **136**: 241–252.

PATENT, D. H., 1970. The early embryology of the basket star *Gorgonocephalus caryi* (Echinodermata, Ophiuroidea). *Mar. Biol.,* **6**: 262–267.

PEARCY, W. G., AND S. W. RICHARDS, 1962. Distribution and ecology of fishes of the Mystic River Estuary, Connecticut. *Ecology,* **43**: 248–259.

RICE, M. E., 1967. A comparative study of the development of *Phascolosoma agassizi, Golfingia pugettensis,* and *Themiste pyroides* with a discussion of developmental patterns in the sipunculia. *Ophelia,* **4**: 143–171.

STANCYK, S. E., 1973. Development of *Ophiolepis elegans* (Echinodermata: Ophiuroidea) and its implications in the estuarine environment. *Mar. Biol.,* **21**: 7–12.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the Sound (Oresund). *Medd. Komm. Dan. Fisk. Havundersog. Copenhagen, Series Plankton,* **4**: 1–523.

WOODLEY, J. D., 1975. The behaviour of some amphiurid brittle-stars. *J. Exp. Mar. Biol. Ecol.,* **18**: 29–46.

Reference: *Biol. Bull.*, **152**: 64–74. (February, 1977)

MORPHOLOGICAL AND CYTOCHEMICAL STUDIES ON THE SECRETORY GRANULES OF THE PYLORIC CAECA OF THE STARFISH, *ASTERIAS AMURENSIS*

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Recently, some extensive biochemical studies on the properties of starfish proteases have been reported. The results have shown that the pyloric caeca of various species of starfish contain some trypsin-like enzymes (Camacho, Brown and Kitto, 1970; Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974, 1975; Elyakova and Kozlovskaya, 1975). These purified enzymes, having a molecular weight of about 25,000, are inhibited by N-a-tosyl-L-lysyl-chloromethane or diisopropylphosphofluoridate and show substrate specificities similar to bovine trypsin. The cleavage specificity and amino acid composition are also strikingly similar to that of bovine trypsin (Gilliam and Kitto, 1976; Camacho, Brown and Kitto, 1976). It is thus highly probable that starfish tryptic enzymes are homologous to vertebrate trypsins.

The starfish proteases have been assumed to be contained in the iron hematoxylin- or azocarmine-positive granules in the secretory cells (Anderson, 1953, 1966; Chia, 1969). However, as far as the authors are aware, there is no positive proof for this assumption. In addition, the ultrastructure of these granules has not been reported. Therefore, the present investigations have been carried out to study the fine structure of the granules and also to clarify whether these granules actually possess proteolytic enzymes in a zymogen form.

Materials and Methods

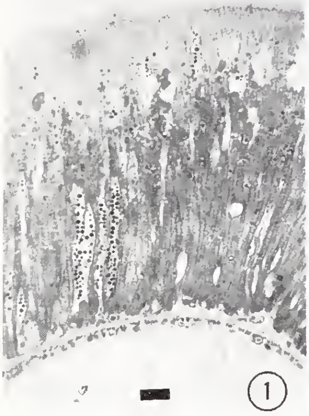
Starfish, *Asterias amurensis*, were collected in the vicinity of Oshoro Biological Station during the period of January to December, 1975.

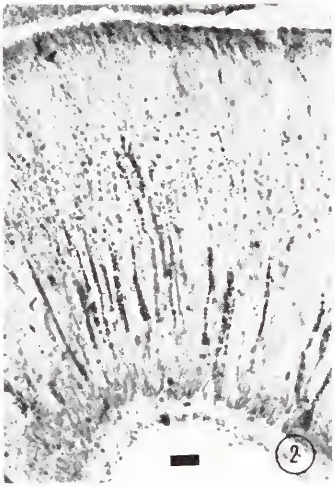
Electron microscopy

Fresh pyloric caeca and the isolated secretory granules were fixed in Karnovsky's glutaraldehyde-formaldehyde fixative (Karnovsky, 1965), post-fixed in 1% OsO_4 buffered with 0.1 M cacodylate, pH 7.4, dehydrated through graded ethanol and embedded in Epon (Luft, 1961) as usual. Ultrathin sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined under a Hitachi HU-12 electron microscope.

Staining of the secretory granules

The one μ thick Epon sections were stained with 0.1% azocarmine-5% acetic acid overnight at room temperature, differentiated with 5% phosphotungstic acid for one hour, rinsed in water and mounted in synthetic resin after drying. With





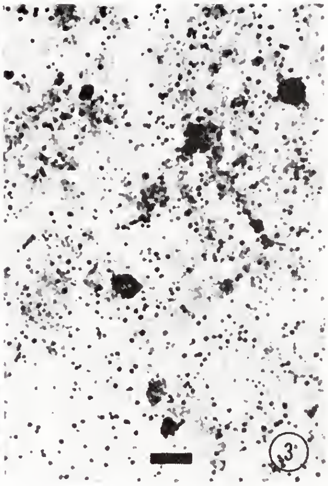


FIGURE 1. Thick Epon section of the pyloric caeca stained with azocarmine, showing the positive reaction of secretory granules. Scale bar is 10 μ.

FIGURE 2. Formalin-fixed, frozen section of the pyloric caeca stained with Baker's acid hematein, showing the positive reaction of secretory granules. Scale bar is 10 μ.

FIGURE 3. Azocarmine-methylene blue staining of the secretory granule-rich (1.7 M) fraction obtained by sucrose density gradient centrifugation of pyloric caeca homogenates. Scale bar is 10 μ.

this technique, only the secretory granules in the secretory cells were stained red (Fig. 1).

Besides the azocarmine method described above, the acid hematein test for phospholipids (Baker, 1946; Hori, 1963) was also found to be specific for the secretory granules (Fig. 2). However, this method was more time-consuming than the azocarmine method.

The fractions obtained during isolation of the secretory granules were smeared on microscope slides, fixed in ethanol-formalin-acetic acid (85:10:5) for ten minutes, rinsed in water and stained with 0.1% azocarmine-5% acetic acid for one hour. The slides were then rinsed in water, differentiated with 5% phosphotungstic acid for fifteen minutes, and stained in a formic acid-sodium acetate buffer containing 0.2% methylene blue (Dempsey and Singer, 1946). After washing in water, the slides were dehydrated and mounted in synthetic resin as usual. With this method, the secretory granules were stained brilliant red, the nuclei pink, and other components blue (Fig. 3).

Protease assay

Samples up to 0.1 ml were incubated in 2 ml of 1% casein in 0.1 M borate buffer, pH 8.5, for fifteen minutes at 30° C. The reaction was stopped by adding 4 ml of 5% trichloroacetic acid (TCA). The mixture was then filtered and optical density of the filtrate was measured at 280 nm (Kunitz, 1947). The blanks were prepared by first mixing casein solution with TCA and then adding enzyme. One unit of casein-hydrolyzing activity was defined as the amount of enzyme which gave rise to an increase in absorbancy at 280 nm of 1.000 per minute under the conditions of assay.

This method was devised according to previous data (Hori, 1975), which indicated that the partially purified proteases had an optimum pH at about 8.5 and that under the above assay conditions, the rate of casein hydrolysis was linear with both time and enzyme concentration when optical density changes of less than 0.05 per minute were measured.

Electrophoresis

Proteases, about 0.02 units, were electrophoresed on polyacrylamide gels by the method of Ornstein (1964) and Davis (1964) using plastic columns, 3 × 12 × 100 mm. After electrophoresis, the gels were sliced into strips of 1.5 mm thickness and placed on the substrate-agar film prepared as follows: 1 g casein, 10 g urea and 1 g agar were dissolved in 100 ml of 0.1 M borate buffer, pH 8.5 by warming. After filtering, 1.5 ml of this solution was spread over a standard microscope slide and allowed to solidify at room temperature.

The slides carrying polyacrylamide gels were incubated in a moist chamber at 30° C for thirty minutes and immersed in acetic ethanol (50% ethanol containing 5% acetic acid). After removal of gels, the substrate-film was air-dried, stained in 0.2% Amido black in acetic ethanol, rinsed in acetic ethanol and air-dried. This is a modification of Uriel's method (1960).

Histochemical detection of the protease activity

The pyloric caeca were fixed in 10% formalin containing 1% $CaCl_2$ for six hours at 4° C, impregnated with 20% sucrose-20% gum acacia overnight and frozen-sectioned in a cryostat. Sections were then washed with cold 95% ethanol, mounted on gelatin film and air-dried. The gelatin film was prepared as follows: 3.5 g gelatin was dissolved in 100 ml of distilled water by warming. Of this solution 0.4 ml was spread over a microscope slide, air-dried, polymerized in 25% formalin overnight and washed thoroughly with running tap water.

Sections mounted on gelatin film were incubated in 50 mM borate buffer, pH 8.5 at 30° C for five to fifteen minutes. After incubation, sections were flashed off the film, and the film was stained first in 1% light green SF for five minutes and then in 0.5% basic fuchsin for twenty minutes. This is a modification of Cunningham's method (1967).

Isolation of the secretory granules

With the azocarmine staining as the marker, the secretory granules were isolated from epithelial cells of the pyloric caeca. Fresh pyloric caeca, 8 g in each experiment, were placed in 70 ml of 1 M sucrose (10 mM $CaCl_2$ was added to all sucrose solutions used in this study). They were dissociated by mincing with scissors and by gentle pipetting. The samples were then filtered through a platinum mesh (150 mesh) and the volume of the filtrate was adjusted to 80 ml with 1 M sucrose. The filtrate was then centrifuged at 4300 × g for ten minutes. The precipitate was washed with 1 M sucrose, suspended in 15 ml of 1.3 M sucrose, sonicated for five seconds and subjected to sucrose density gradient centrifugation; each centrifugation tube contained from bottom to top 1 ml of 1.8 M sucrose, 1.3 ml of 1.7 M sucrose and 2.5 ml of sample in 1.3 M sucrose. The samples were centrifuged at 40,000 rpm (maximum, 175,000 × g; minimum, 84,000 × g) for one hour using a Hitachi swinging bucket rotor RPS 50. After centrifuging, the content of each tube was divided into supernatant, 1.3 M (interphase between 1.3 M and 1.7 M layers), 1.7 M and precipitate fractions. The 1.7 M fraction contained the majority of secretory granules (Fig. 3).

For protease assay and electrophoresis, each fraction was homogenized and placed at 37° C for one hour in order to activate proteases. For protein assay, each fraction was diluted with an appropriate amount of water, and treated with 5% TCA. The TCA precipitate was taken up with 0.1 N NaOH and assayed by the method of Lowry, Rosebrough, Farr and Randall (1951).

RESULTS

Ultrastructure of the secretory granules

The azocarmine-positive granules, measuring 0.5–2.0 μ in diameter, are limited by a single membrane measuring about 60 Å in thickness and may be classified into three types according to the morphology of their contents: first, dense granules which contain dense, homogeneous amorphous substances; secondly granules which contain a dense, homogeneous core imbedded in amorphous substances, and thirdly, light granules which contain amorphous substances only (Figs. 4–7). The size and shape of the core in the second-type granules varied greatly in different granules. This seems to suggest that these granules might be transitory forms from the light to dense granules (Fig. 7). The dense granules may be considered the matured forms.

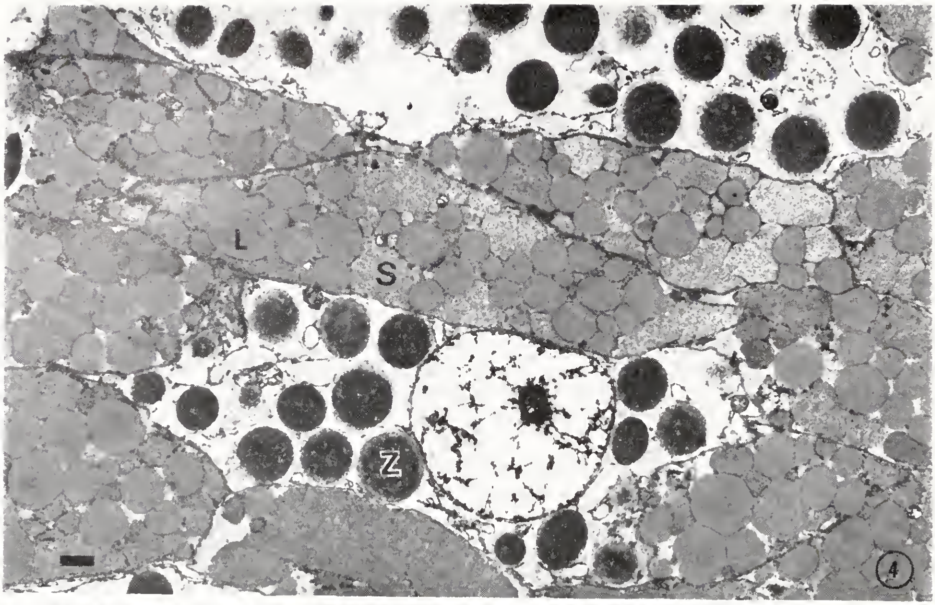
The light granules were much more numerous than the dense granules in the specimens collected during the pre-breeding season (January to May), while the dense granules were predominant in the specimens collected during the post-breeding season (August to December). All types of granules were positively stained with azocarmine.

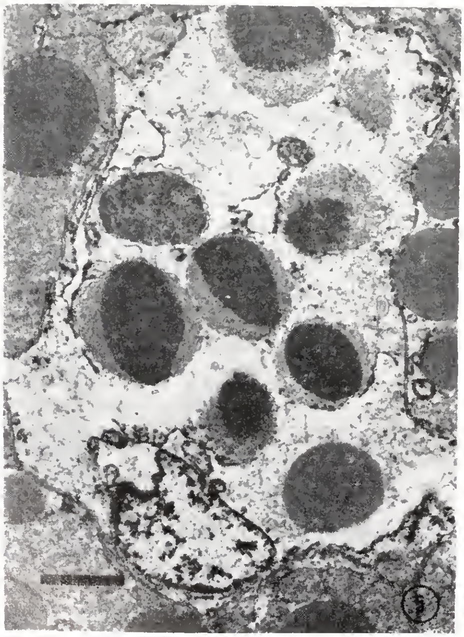
The granules were often seen abutted on rough endoplasmic reticulum, but the membrane of granules was in no case continuous with the membrane of rough endoplasmic reticulum. Golgi apparatuses were not numerous.

The clear vacuole, usually associated with the secretory granule clump (Anderson, 1953, 1966), appeared to be either an empty space formed by an extensive outgrowth of the outer nuclear membrane or a large space which was limited by a single membrane, but contained little or no electron-dense material (Fig. 7). The cytoplasm of the secretory cell was rather scanty in comparison with the adjacent storage cells, but the rough endoplasmic reticulum was well-developed, particularly in the middle portion of the cell.

Histochemical detection of the protease activity

With the substrate-film method, the protease activity was demonstrated in the glandular epithelium as well as in the lumen. Because of the poor resolution inherent to such methods, it was impossible to ascertain if the intraepithelial activity was actually located specificially in the secretory granules.





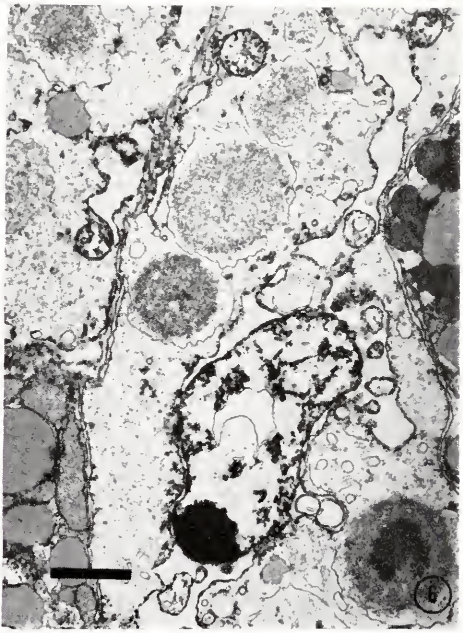


FIGURE 4. Fine structure of the secretory cells, showing dense secretory granules (Z); L and S, lipid and storage granules in the adjacent storage cell. Scale bar is 1 μ.

FIGURE 5. Fine structure of the secretory granules which have a dense core surrounded by light amorphous substance. Scale bar is 1 μ.

FIGURE 6. Fine structure of the secretory granules which are characterized by light amorphous content. Note an extensive outgrowth of the outer nuclear membrane studded with ribosomes. Scale bar is 1 μ.

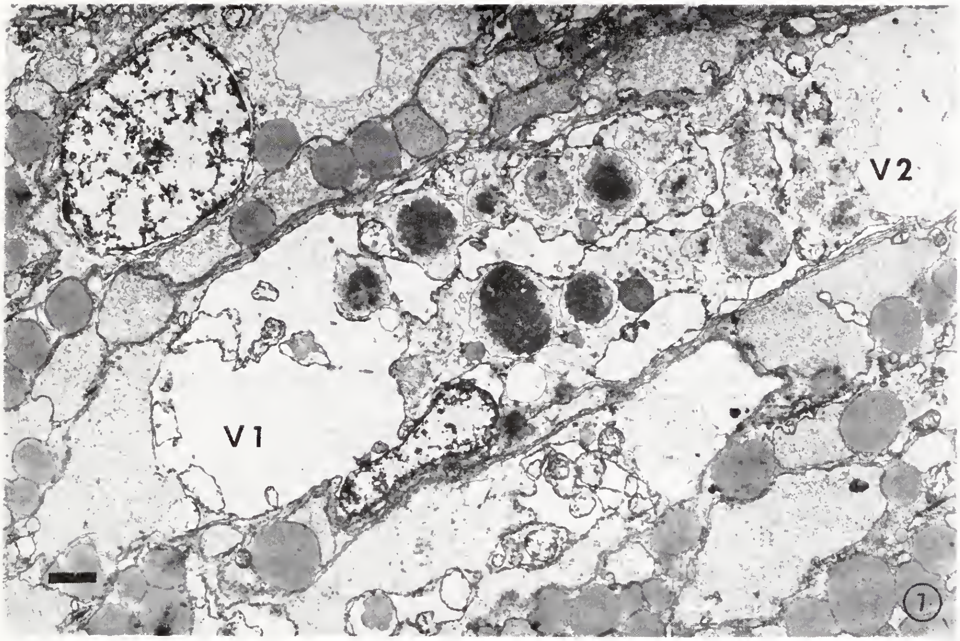


FIGURE 7. Fine structure of a secretory cell containing several secretory granules of different types, in addition to the associated vacuoles, V_1 and V_2. Scale bar is 1 μ.

Properties of the isolated secretory granules

The results of the enzyme and protein assays of the various fractions obtained during one purification procedure are given in Table I. The isolation was performed six times with six different animals, and the results were essentially the same.

TABLE I

Separation of secretory granules from pyloric caeca.

	Tissue homogenate	4300 × g sediment	Density gradient				
			Supernatant	1.3 M	1.7 M	Sediment	Total
Protease activity (units/g tissue)	13.04	6.07	0.76	1.27	2.29 *s 1.78 *p 0.51	0.44	4.76
Protein (mg/g tissue)	84.97	11.78	0.85	5.00	1.70 *s 0.34 *p 1.36	3.78	11.33
Specific activity (units/mg protein)	0.15	0.52	0.89	0.25	1.35 *s 5.24 *p 0.38	0.12	

*s and *p, 27,000 × g supernatant and sediment of the homogenized 1.7 M fraction, respectively.

The amount of azocarmine-positive granules estimated by histological inspection was in agreement with the quantitative data on the protease activity; *i.e.*, the granules were much more abundant in the 1.7 M fraction than in the other fractions (Table I). About 50% of the total enzyme activity was recovered in the 1.7 M fraction. The relatively high specific activity of proteases in the supernatant fraction would be due to the enzyme released from the granules during centrifugation.

Attempts to further purify the granules by subjecting the 1.7 M fraction to the second sucrose density gradient centrifugation were fruitless, probably because of the fragility of granules. In fact, more than 50% of the activity became unsedimentable, and the latency of the protease activity in the sedimented granules was lost after the second centrifugation.

The 1.7 M fraction exhibited a very low level of enzyme activity, if assay was

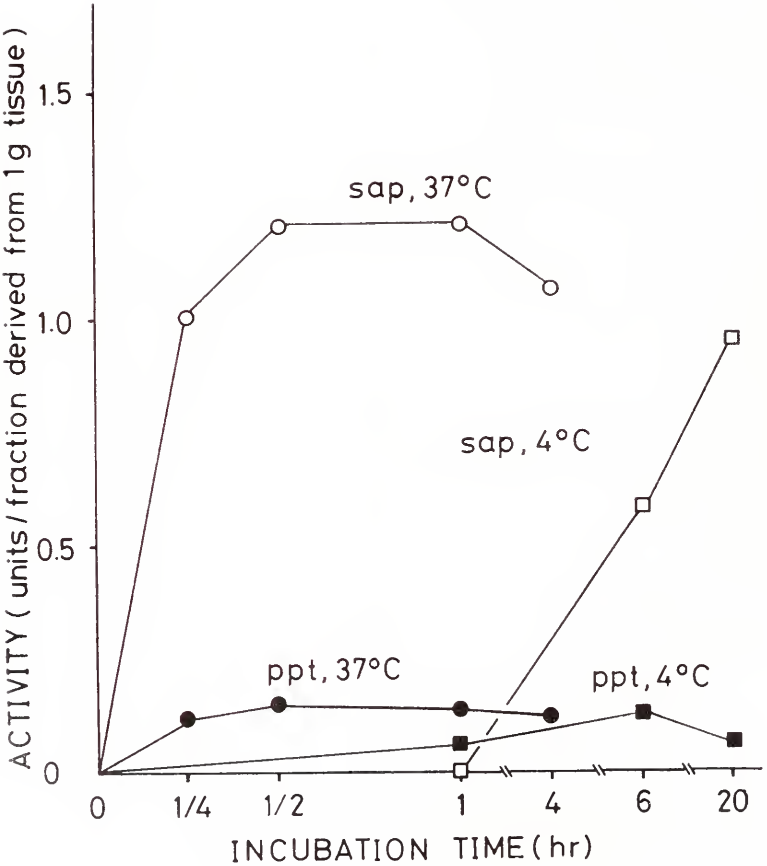


FIGURE 8. Activation of proteases by incubation. The 1.7 M fraction was homogenized, separated into supernatant (sap) and sediment (ppt) by centrifuging at 27,000 × g for five minutes, and incubated at 4° C or at 37° C. Protease activity is expressed as units per fraction derived from g tissue.

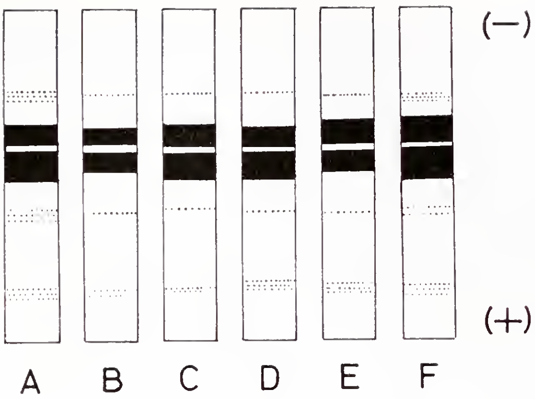


Figure 9. Electrophoretic separation of the casein-hydrolyzing activities: a, pyloric caeca homogenates; b, 4300 × *g* sediment of the homogenates; c-f, supernatant, 1.3 M, 1.7 M and sediment fractions obtained by sucrose density gradient centrifugation of the 4300 × *g* sediment, respectively. No significant change in the electrophoretic pattern occurred during separation of the secretory granules.

performed with the freshly prepared fraction. On the other hand, the enzyme was activated rapidly by incubation at 37° C. The activation also occurred at 4° C, though it required longer duration (Fig. 8). This suggests that the enzyme activation may be physiologically possible in poikilothermal animals. The results presented in Figure 8 also show that solubilization of the enzymes by homogenization alone would not cause their instantaneous activation, that the activation might be an enzyme-catalyzed reaction, and that the enzymes exist in easily-solubilized forms and are not bound to the granular membranes.

The specific activity of the 1.7 M fraction was nine times, and that of the supernatant obtained from the 1.7 M fraction was thirty-five times greater than that of the original tissue homogenates (Table I). Electrophoretic patterns of the activated enzymes were almost the same in all fractions obtained during purification (Fig. 9). The data excluded the possibility that particular forms of the enzymes were selectively isolated during purification.

By electron microscopy, the 1.7 M fraction contained numerous granules, the morphology of which were the same as those of the secretory granules in tissues. Some contaminated elements, such as smooth-surfaced vesicles, mitochondria and storage granules usually appeared in these fractionated specimens. Many secretory granules were present separately, but some were isolated in the form of clumps, surrounded by dense cytoplasm rich in rough endoplasmic reticulum (Fig. 10). Mitochondria were often observed between such clumps of granules.

Discussion

The ultrastructure of secretory granules in the starfish pyloric caeca has been described probably for the first time in the present paper. In a report on the ultrastructure of the pyloric caeca of *Asterias rubens*, Bargmann and Behrens (1968) have mentioned that the so-called zymogen granules have not been observed.

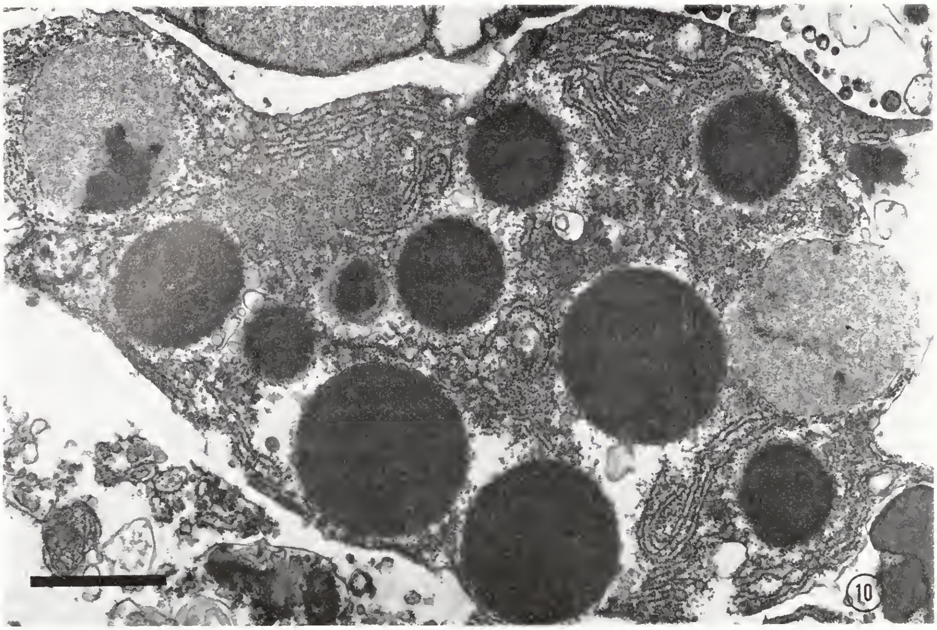


FIGURE 10. Fine structure of an aggregate of the secretory granules found in the 1.7 M fraction obtained by sucrose density gradient centrifugation, showing the secretory granules of dense, light and core-bearing types as well as the surrounding rough-surfaced endoplasmic reticulum. Scale bar is 1 μ.

Their failure in identifying the granules might be related to the morphological variation of the ultrastructure of granules, because secretory granules in starfish pyloric caeca do not always appear as dense as those in mammalian pancreatic acini. As described above, some granules (light granules) are characterized by amorphous contents which (like the dense granules) are also azocarmine-positive. Therefore, if the specimens from the pre-breeding season were used for study, then the light granules could predominate. It would be difficult to identify the granules as zymogen morphologically, unless a careful comparison was made from light- and electron microscopic preparations.

Morphologically, the dense granules appear to be the mature form and the granules having a dense core appear to be transitory forms between the light and dense granules. However, it is also possible that some core-bearing granules might have already been matured. Possibility also exists that the morphological heterogeneity of the granules might reflect their biochemical heterogeneity (*i.e.*, different types of granules contain different sets of hydrolytic enzymes). The granular fraction obtained by sucrose density gradient centrifugation exhibited the same electrophoretic pattern of protease activity as that of the original tissue homogenates. However, this does not necessarily mean that each isolated granule is biochemically equivalent.

The dense granules in starfish pyloric caeca were morphologically quite similar to the secretory granules described by Holland and Lauritis (1968) in gastric exocrine cells of the purple sea urchin, *Strongylocentrotus purpuratus*. It is uncertain, however, if the gastric exocrine cells also contain granules homologous to the light and core-bearing granules described in the present paper.

It was extremely difficult to obtain a pure fraction of the secretory granules, because of the wide spectrum of the density of the granules. The same was true with other cellular organelles, such as mitochondria and rough and smooth endoplasmic reticulum. Our attempts to obtain pure fractions of these organelles by modifications of the methods commonly employed for fractionation of vertebrate tissues have so far been unsuccessful. Obviously, an entirely different technique is required for such works with starfish pyloric caeca.

It has been reported that the proteases in starfish pyloric caeca are stored as inactive zymogens (Camacho, Brown and Kitto, 1970; Bundy and Gustafson, 1973), and that the zymogen may be contained in granules (Peng and Williams, 1973; Tillinghast and Levasseur, 1975). Such reports are based on the findings that the protease activity of crude homogenates or of an extract of acetone powder increases upon incubation at 20° C or above, and that the activity in crude homogenates can be easily sedimented by relatively low centrifugal force. The present data not only are consistent with these observations, but also have clearly demonstrated that the enzymes are present in the so-called zymogen granules in an inactive form. These enzymes may be activated by self-catalyzed hydrolysis or contact with some activators or removal of inhibitors. The present data do not agree with the findings of Tillinghast and Levasseur (1975), who state that when the enzyme is spontaneously released from the granules, further activation does not occur. Presumably, in the pyloric caeca, the content of the secretory granules is released into the glandular lumen, where activation of proteases may take place as with mammalian enzymes.

We are grateful to Professor Jeffrey P. Chang for reading the manuscript and correcting the English.

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SUMMARY

A fraction rich in secretory granules was prepared from the pyloric caeca of *Asterias amurensis* by sucrose density gradient centrifugation. The freshly prepared fraction exhibited no casein-hydrolyzing activity, but showed nine times as much specific activity as that of tissue homogenates after incubation at 37° C for thirty minutes. Electron microscopy showed that the secretory granules were membrane-bound granules measuring 0.5–2.0 μ in diameter and contained dense and/or light amorphous substances.

LITERATURE CITED

ANDERSON, J. M., 1953. Structure and function in the pyloric caeca of *Asterias forbesi*. *Biol. Bull.*, **105**: 47–61.

ANDERSON, J. M., 1966. Aspects of nutritional physiology. Pages 329–357 in R. A. Boolootian, Ed., *Physiology of Echinodermata*. John Wiley and Sons, New York.

Baker, J. R., 1946. The histochemical recognition of lipine. *Quart. J. Microsc. Sci.,* **87**: 441–471.

Bargmann, W., and B. Behrens, 1968. Über die Pylorusanhänge des Seesterns (*Asterias rubens* L.), insbesondere ihre Innervation. *Z. Zellforsch.,* **84**: 563–584.

Bundy, H. F., and J. Gustafson, 1973. Purification and comparative biochemistry of a protease from the starfish, *Pisaster giganteus. Comp. Biochem. Physiol.,* **44**: 241–251.

Camacho, Z., J. R. Brown, and G. B. Kitto, 1970. Purification and properties of trypsin-like proteases from the starfish, *Dermasterias imbricata. J. Biol. Chem.,* **245**: 3964–3972.

Camacho, Z., J. R. Brown, and G. B. Kitto, 1976. Structural studies on a starfish trypsin. *Comp. Biochem. Physiol.,* **54B**: 27–32.

Chia, F. S., 1969. Histology of the pyloric caeca and its changes during brooding and starvation in a starfish, *Leptasterias hexactis. Biol. Bull.,* **136**: 185–192.

Cunningham, L., 1967. Histochemical observation of the enzymatic hydrolysis of gelatin films. *J. Histochem. Cytochem.,* **15**: 292–298.

Davis, B. J., 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.,* **121**: 404–427.

Dempsey, E. W., and M. Singer, 1946. Observations on the chemical cytology of the thyroid gland at different functional stages. *Endocrinology,* **38**: 270–295.

Elyakova, L. A., and E. P. Kozlovskaya, 1975. Proteinases of starfishes-I. *Comp. Biochem. Physiol.,* **50B**: 249–253.

Gilliam, E. B., and G. B. Kitto, 1976. Isolation of a starfish trypsin by affinity chromatography. *Comp. Biochem. Physiol.,* **54B**: 21–26.

Holland, N. D., and J. A. Lauritis, 1968. The fine structure of the gastric exocrine cells of the purple sea urchin, *Strongylocentrotus purpuratus. Trans. Amer. Microsc. Soc.,* **87**: 201–209.

Hori, S. H., 1963. A simplified acid hematein test for phospholipids. *Stain Technol.,* **38**: 221–225.

Hori, S. H., 1975. Proteolytic enzymes from the pyloric caeca of the starfish, *Asterias amurensis. Acta Histochem. Cytochem.,* **8**: 80.

Karnovsky, M. J., 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.,* **27**: 137A–138A.

Kozlovskaya, E. P., and L. A. Elyakova, 1974. Purification and properties of trypsin-like enzymes from the starfish, *Lysastrosoma anthosticta. Biochim. Biophys. Acta,* **371**: 63–70.

Kozlovskaya, E. P., and L. A. Elyakova, 1975. Certain properties of preparations from starfish proteinases-II. *Comp. Biochem. Physiol.,* **50B**: 255–258.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor-II. General properties. *J. Gen. Physiol.,* **30**: 291–320.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.,* **193**: 265–275.

Luft, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.,* **9**: 409–414.

Ornstein, L., 1964. Disc electrophoresis-I. Background and theory. *Ann. N. Y. Acad. Sci.,* **121**: 321–349.

Peng, R. K. Y., and D. C. Williams, 1973. Partial purification and some enzymatic properties of proteolytic enzyme fractions isolated from *Pisaster ochraceus* (Echinoderm) pyloric caeca. *Comp. Bochem. Physiol.,* **44B**: 1207–1217.

Reynolds, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.,* **17**: 208–212.

Tillinghast, E. K., and S. Levasseur, 1975. Protease activation in the starfish *Asterias forbesi. Comp. Biochem. Physiol.,* **50B**: 131–135.

Uriel, J., 1960. A method for the direct detection of proteolytic enzymes after electrophoresis in agar gel. *Nature,* **188**: 853–854.

Watson, M. L., 1958. Staining of tissue sections for electron microscopy with heavy metals. *J. Biophys. Biochem. Cytol.,* **4**: 475–478.

Winter, W. P., and H. Neurath, 1970. Purification and properties of a trypsin-like enzyme from the starfish, *Evasterias trochelii. Biochemistry,* **9**: 4673–4679.

Reference: *Biol. Bull.*, **152**: 75–83. (February, 1977)

DEVELOPMENT OF THE DIMORPHIC CLAW CLOSER MUSCLES OF THE LOBSTER *HOMARUS AMERICANUS*: I. REGIONAL DISTRIBUTION OF MUSCLE FIBER TYPES IN ADULTS

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An interesting feature of a number of crustaceans is the fact that their claws (first periopods) are dimorphic. This is particularly evident in species such as male fiddler crabs (*Uca*, sp.), the pistol shrimp (*Alpheus*, sp.) and the lobster (*Homarus*, sp.) (Przibram, 1931). However, detailed physiological studies of the claw muscles of these animals seem to be limited to the last named species. In an early study of *H. vulgarus*, Wiersma (1955) suggested that the shorter, stout crusher claw was only capable of closing slowly. In contrast, the longer, narrow cutter claw was capable of closing very rapidly. More recent work (Jahromi and Atwood, 1971a; Goudey and Lang, 1974) in *H. americanus* suggested that this was probably a result of the differential distribution of muscle fibers in the closer muscles of the two claws. The cutter claw closer muscle had two populations of muscle fibers, short sarcomere (< 4 μm) fast fibers and long sarcomere (> 6 μm) slow fibers. The adult crusher claw had primarily long sarcomere (> 6 μm) slow fibers and a few intermediate fibers (4–6 μm). Although the division of crustacean skeletal muscle into short sarcomere fast fibers and long sarcomere slow fibers has been well established (Atwood, 1972), an exceptional example has been noted in a crab (Hoyle, 1973). In the present case, the dicotomy seems justified on the basis of physiological studies on single fibers (Jahromi and Atwood, 1971a) and on intact closer muscles of the lobster (Govind and Lang, 1974).

The dimorphism in the claws of the lobster is not evident in the early developmental stages (up to stage 6 or 7) when both claws resemble cutter claws in external morphology (Herrick, 1896). Whether the muscle fiber types and their distribution patterns are also identical in the two claws remains to be shown. Certainly a distinct crusher has not yet differentiated. Even when one of the pair does eventually differentiate into a crusher, it still resembles the cutter somewhat be possessing some fast fibers in the juvenile stages (Goudey and Lang, 1974). Therefore, since dimorphism in the lobster claws appears well after the early juvenile stages, it affords a unique opportunity to trace the development of fiber types in the closer muscles.

The present paper establishes the distribution of muscle fiber types (on the basis of sarcomere lengths) in a range of adult cutter and crusher closer muscles. Subsequent papers will trace the development of these adult patterns by examining larval and postlarval (juvenile) stages.

MATERIALS AND METHODS

Lobsters (*Homarus americanus,* Milne-Edwards) were trapped in the local waters around Woods Hole, Massachusetts and kept in ambient running sea water. One large animal (3600 g) was purchased from a local supplier. All animals were held without claw restraints and were fed several times weekly. Animals were weighed several minutes after being taken out of the sea water. No attempt was made to dry them completely. Lengths were measured from the tip of the rostrum to the end of the telson.

The closer muscles were prepared in a manner similar to that used by Jahromi and Atwood (1971a). Closer muscles were immobilized by clamping the dactyl in the open position. Perfusion was then accomplished by inserting hypodermic needles through holes in the exoskeleton to ensure delivery of Bouin's fixative to all parts of the muscle. After perfusing for 2–3 hr, the closer muscle was exposed by chipping away the exoskeleton and removing the opener muscle. The claw was subsequently immersed in fresh fixative for several hours. After fixation, the closer muscle was removed and stored in 90% ethanol.

To provide a basis on which to sample the closer muscle, the inner aspect of each muscle was divided into nine sections and at least 10 fibers sampled from each section. Previous work had shown the outer aspect to have a similar distribution of fiber types (unpublished observations). Proximal, central and distal areas were identified and each was further subdivided into dorsal, medial and ventral sections, giving a total of nine areas to sample (Fig. 1). Fibers from each section were put into individual vials filled with 90% ethanol. In order to examine the possibility that fiber types were distributed homogeneously within areas, a different sampling procedure was used for the closer muscles of the largest animal (3600 g). Fibers were removed from the outer edges of all areas except the central medial area, from which the center fibers were sampled (hatched areas, Fig. 1). Thus in the dorsal section, only the most dorsal fibers were sampled, and similarly, only the most ventral fibers in the ventral

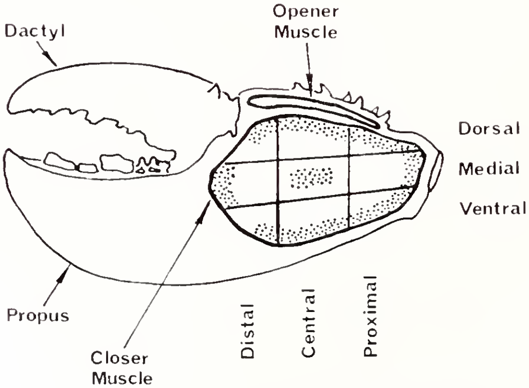

FIGURE 1. Inner aspect of a crusher claw showing the closer muscle subdivided into nine sections for sampling of fiber types. The stippled portions depict the areas sampled in the 3600 g animal.

TABLE I

Per cent distribution of muscle fiber types in the dimorphic claw closer muscles of lobsters.

Animal (number)	Length (cm)	Weight (g)	Muscle fiber types based on sarcomere length (μm)							
			Cutter				Crusher			
			N	4 (Fast)	4–6 (Intermediate)	6 (Slow)	N	4 (Fast)	4–6 (Intermediate)	6 (Slow)
1	13	100	185	63%	9	37%	180	0	0	100%
2	15.5	160	108	71	2	27	108	0	0	100
3	24	416	180	63	0	37	180	0	4	96
4	30.4	3600	97	55	0	45	50	0	0	100

N = Total number of fibers sampled in each claw.

section. The technique reduced the sampling overlap between adjacent sections and gave an indication of the homogeneity of fiber types within a restricted area.

To determine sarcomere lengths, individual muscle fibers were teased apart in 90% ethanol on a glass microscope slide and measured using a compound microscope equipped with a filar micrometer eyepiece. The average sarcomere length of a fiber was obtained by measuring five consecutive sarcomeres in each of three myofibril bundles within a fiber. Errors due to local contraction or damage were compensated by eliminating any fiber in which sarcomere length for individual myofibrils differed by more than 20%, the amount of variability which has been shown to occur naturally in crab muscle fibers (Franzini-Armstrong, 1970).

RESULTS

One of the problems encountered in this and other studies was the unequivocal identification of single muscle fibers. When fibers were removed from the closer muscles, they were generally present in "units" (Atwood, 1972; Jahromi and Atwood, 1971b). These were placed *in toto* in vials. When units were subdivided, it was invariably noted that adjacent fibers were attached to each other with connective tissue strands. In addition, cytoplasmic bridges were often observed joining adjacent fibers (Jahromi and Atwood, 1971b; Goudey and Lang, 1974). The criterion for fiber isolation was to subdivide units as much as possible without shredding the membranes, even if this meant cutting small cytoplasmic bridges. Thus, even though fibers might actually be morphological subunits, the small bridges represented high resistance electrical pathways (Jahromi and Atwood, 1971b) that would probably serve to render fibers physiologically independent in terms of contractile activation by motor nerves.

Muscle fiber types in the cutter claw

The claw closer muscle was examined in four lobsters with the following total weights, 100 g, 160 g, 416 g, and 3600 g. The distribution of muscle fiber types from the inner aspect of the cutter claws did not differ markedly among the four claws examined. In general, most fell into two categories, the majority

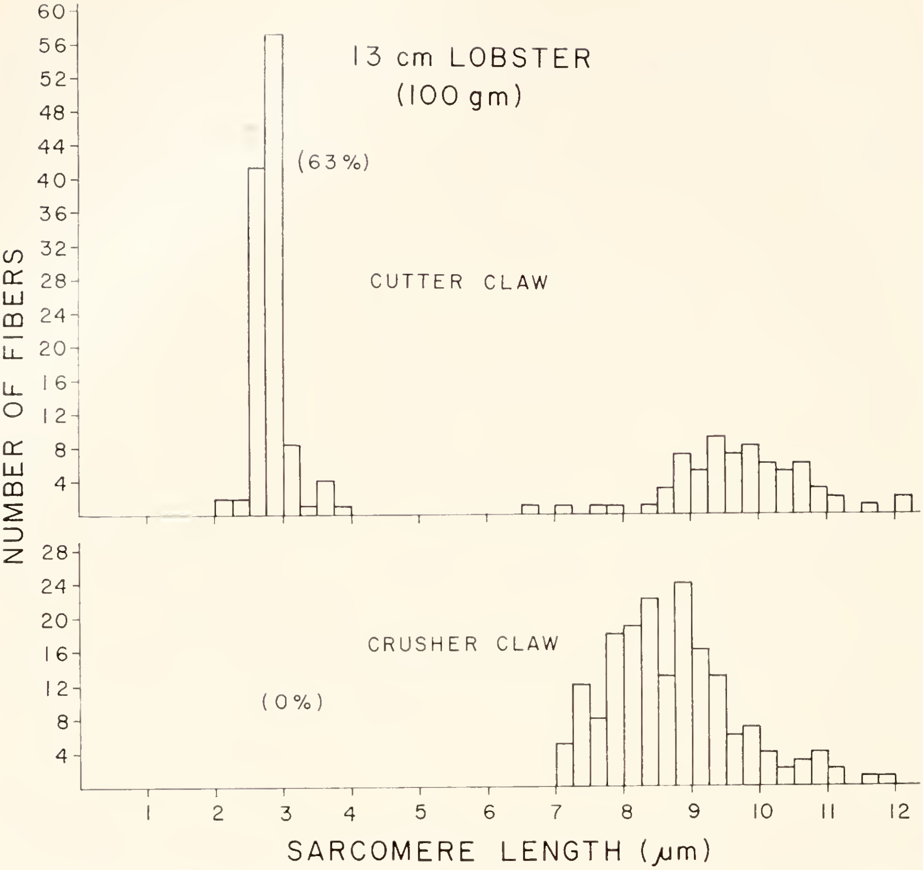

FIGURE 2. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of the cutter and crusher closer muscles of a 100 g lobster. The proportion of fast fibers is shown.

being short sarcomere (2–4 μm) fast fibers and the remainder longer sarcomere (6–12 μm) intermediate and slow fibers (Table I, Fig. 2). For the three smaller animals, the fast fibers composed 63–68% of the fiber population while the largest animal had 55%. This discrepancy may simply be due to the sampling method employed for the 3600 g animal (see Methods).

While the four animals did not exhibit any striking differences among their fiber populations, it was apparent that both the smallest and largest animals did not have many intermediate fibers (sarcomeres between 4 μm and 6 μm). In addition, the fast fibers in the largest animal (3600 g) exhibited a tendency for a bimodal distribution with peaks at 2 μm and 3.5 μm.

Regional distribution in the cutter claw

The pattern of distribution of closer muscle fibers in the cutter claw suggested that there is a regional distribution of fast and slow fibers. Representative examples from the smallest (100 g) and largest (3600 g) lobsters are given in Figures 3 and 4. In general, dorsal fibers were uniformly fast when only the most dorsal two or three layers were sampled. This was apparent in the 100 g (Fig. 3) and 205 g animals, where the claws were of a size in which the dorsal section included only fast fibers. The central dorsal and central medial areas never contained slow fibers in any animals examined. Likewise, the ventral fibers tended to be uniformly slow fibers. This was evident in the 100 g and 160 g animals. In the former, only 4 out of the 60 fibers were slow while in the latter there were no slow fibers at all. In the 3600 g animal (Fig. 4), where only the ventral-most fibers were sampled in the ventral section, there were no fast fibers.

The pattern that emerges seems to be the following: fast fibers predominate in the three dorsal sections as well as in the central medial section; slow fibers predominate in the ventral sections. The proximal medial and distal medial sections are mixed and, depending perhaps either on the animal or on the sampling technique, one or the other can predominate. Within these regions, however, the fast and slow fibers seem to be separated into distinct bundles. When only

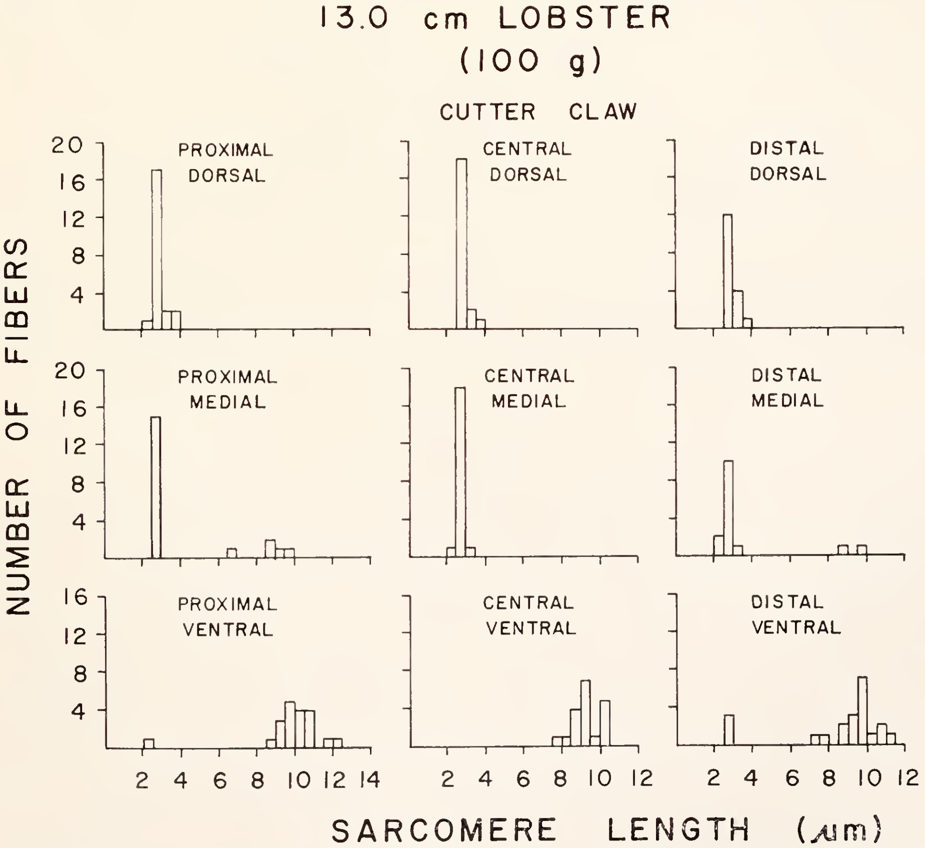

FIGURE 3. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution on the inner aspect of a cutter closer muscle in a 100 g lobster.

the extreme fibers of the sections are sampled, *e.g.*, in the 3600 g animal, a uniform population of fibers was observed for each area.

Muscle fiber types in the crusher claw

The closer muscles of adult (700 g) lobster crusher claws have previously been shown to be composed of intermediate and slow fibers (Jahromi and Atwood, 1971a; Goudey and Lang, 1974). Similar results were obtained in the present study for animals as small as 100 g (Fig. 2). Virtually all crusher fibers had sarcomere lengths greater than 6 μm with the longest generally around 12–13 μm. Only in the 416 g lobster were there some fibers with sarcomere lengths between 5–6 μm. However, its entire crusher fiber population seemed to be shifted to the left as compared to the others.

Regional distribution in crusher claws

Muscle fibers in the crusher claw closer muscles were all intermediate and slow; thus any regional differences, if present, would not be as striking as in the cutter claw. In fact the crusher closer muscle did not show a regional distribution

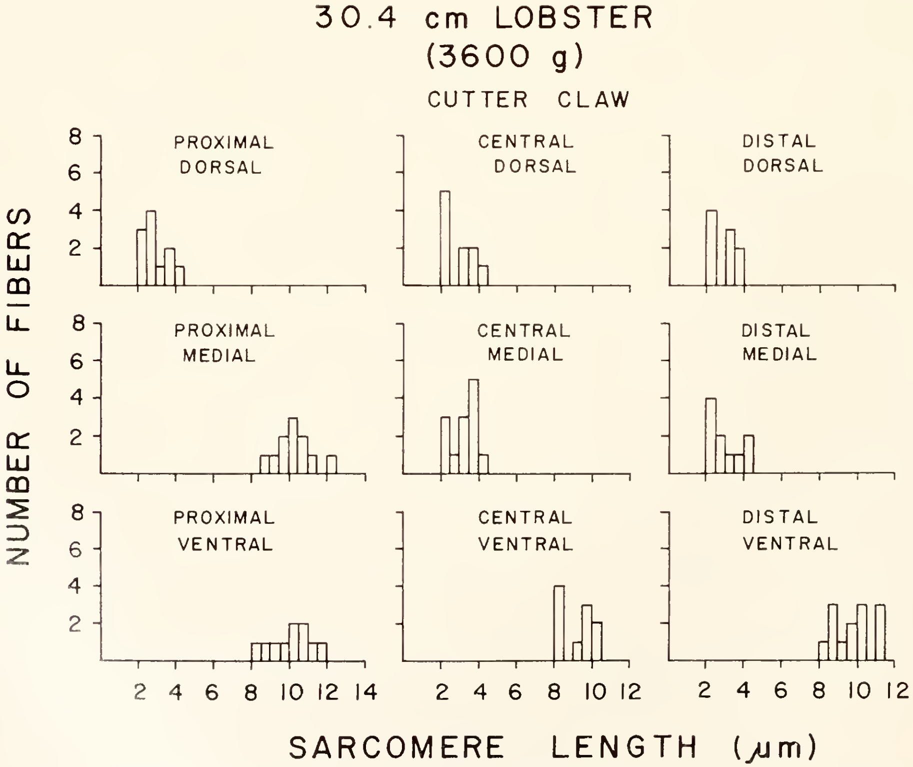

FIGURE 4. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution on the inner aspect of a cutter closer muscle in a 3600 g lobster.

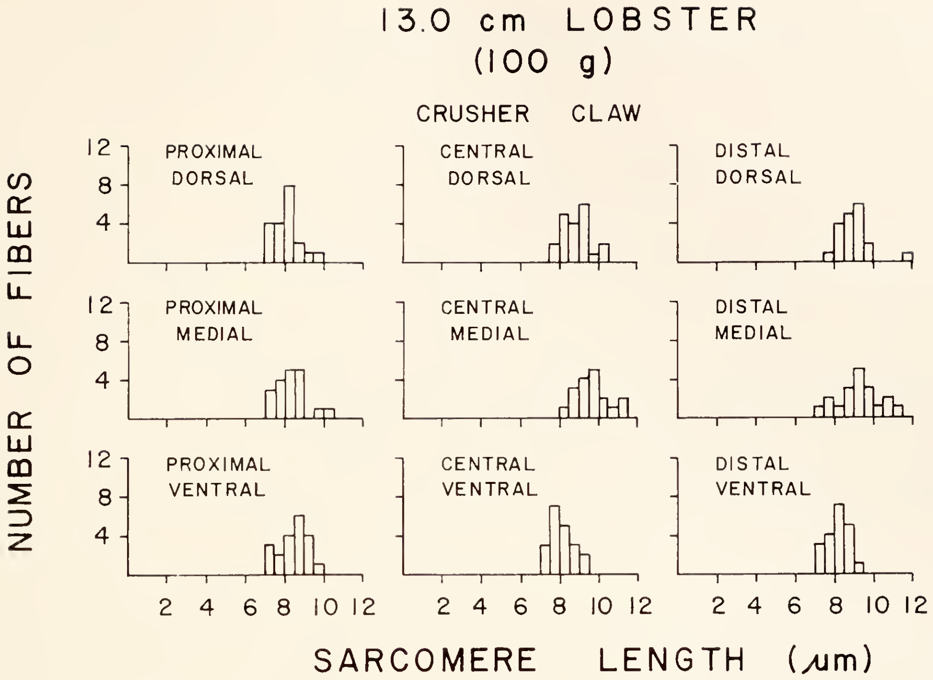

FIGURE 5. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing their mixed distribution on the inner aspect of a crusher closer muscle in a 100 g lobster.

of fast and slow fibers in any of the claws examined (Fig. 5). In some animals there was a tendency for certain areas to have more intermediate fibers than other areas, but this was not uniform among the claws studied.

DISCUSSION

The closer muscles of the lobster cutter claw had been shown to be composed of short sarcomere (2–4 μm) fast fibers and longer sarcomere (6–13 μm) intermediate and slow fibers (Jahromi and Atwood, 1971a; Goudey and Lang, 1974). It has now been established that there is a regional distribution of these fibers with the former present in the dorsal sections as well as the central medial section (Fig. 1). Slow fibers comprise the bulk of the ventral region of the muscle. More recent work has led to the conclusion that the cutter closer muscle is divisible into distinct bundles or groups of fibers. These groups each appear to be composed of a homogenous population of fast or slow muscle fibers (Costello, Govind, She and Lang, 1976).

The crusher claw closer muscle has only intermediate and slow muscle fibers. Within these categories, there does not seem to be a regional distribution among the areas of the crusher claw. However, the crusher claw closer muscle also appears to be divisible into distinct bundles. It is possible therefore that intermediate fibers might be present in certain bundles and not in others.

One problem raised by this study is in regard to the pattern of innervation of the muscle fibers. Both claw closer muscles receive two motor axons, a fast and a slow. In the cutter claw the fast muscle fibers are present in the dorsal area; this area receives innervation almost exclusively from the fast motor axon (Govind and Lang, 1974; unpublished observations). It seems likely, then, that the slow axon might preferentially innervate the more ventral slow fibers in the cutter claw. However, there are a number of fibers, perhaps 20%, that receive innervation from both axons (Govind and Lang, 1974). One might expect that these would be intermediate fibers with sarcomere lengths in the range of 4–6 μm. Such fibers, however, were almost totally absent in the claws examined, except in the 160 g animal. Indeed, the 3600 g animal had no fibers in the cutter claw between 4.5 μm and 8 μm. The nature of these dually innervated fibers remains to be investigated. The presence of the intermediate fibers in the cutter claw might be related to factors other than innervation. For instance, it has been shown that certain lobster muscle fibers grow in length by addition of sarcomeres (Govind, She and Lang, 1977). This seems to be a likely explanation of growth in the closer muscle as adult sarcomere lengths are established early in the life cycle (Goudey and Lang, 1974). Depending on when in the intermolt cycle the fibers add sarcomeres, *i.e.*, immediately before or after the molt, the average sarcomere length may be shorter or longer for these fibers. This problem could profitably be studied by sampling animals at various stages in the molt cycle. It would also be of interest to study the problem in regenerating claws which grow disproportionately faster than the rest of the animal. Here, the muscle fibers would be growing rather rapidly in length.

Another problem raised by this study is in regard to the distribution of the intermediate "fast follower" (Jahromi and Atwood, 1971a) fibers in the crusher claw. Since the crusher claw closer muscle also received two motor axons, a phasic and a tonic (Govind and Lang, 1974), it was expected that the fast axon might preferentially innervate these fibers. Previous work had shown that stimulation of the fast axon could evoke a small twitch in the crusher closer muscle (Govind and Lang, 1974). If this is the case, the intermediate "fast followers" do not appear to be regionally distributed as are the fast fibers of the cutter claw. However, this closer muscle, like that of the cutter claw, also appears to be divisible into distinct bundles. Whether any of these are composed of a homogenous population of intermediate fibers is as yet uncertain.

One of the problems that remains unsettled is how the claws change from a symmetrical condition in the larval and early postlarval stages to the dimorphic condition in the adult. The lobster, after hatching, immediately molts into stage 1, a pelagic mysis larva. It then passes through two more mysis stages and finally becomes a diminutive adult at stage 4. The claws are indistinguishable until stages 6 or 7, at which time the cutter claw is slightly longer and thinner than the crusher (Herrick, 1896, 1911; Emmel, 1908). At stage 4 the closer muscles of both claws contain 35–45% fast fibers while the rest are slow fibers (in preparation). However, at stage 6 or 7, the presumptive cutter claw usually has over 60% fast fibers while the crusher has under 40% fast fibers. These fast fibers in the crusher may be present at least until the 16th stage (Goudey and Lang, 1974). The time course of this change from a symmetrical to an asymmetrical condition will be the subject of subsequent papers in this series.

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Summary

1. The closer muscles of the dimorphic claws (chelipeds) were studied for the presence and location of fast and slow muscle fibers.

2. Cutter claws were composed of about 60–70% short sarcomere (< 4 μm) fast fibers; the remainder was longer sarcomere (> 6 μm) slow and intermediate (4–6 μm) fibers.

3. Crusher claws were composed of a uniform population of long sarcomere (6–13 μm) slow and intermediate (4–6 μm) fibers.

4. There was a regional distribution of fibers in the cutter claw. Ventral fibers were predominantly slow. Dorsal fibers and central medial fibers were fast. Proximal and distal fibers in the medial section were usually mixed.

5. The regional distribution of cutter fibers correlates with previous physiological studies on the distribution of the fast and slow motor axons to these muscle fibers.

LITERATURE CITED

Atwood, H. L., 1972. Crustacean muscle. Pages 421–489 in G. Bourne, Ed., *The structure and function of muscle, Vol. 1, 2nd Ed.* Academic Press, New York.

Costello, W. J., C. K. Govind, J. She, and F. Lang, 1976. Morphology of the closer muscles during the growth of the lobster *Homarus americanus*. *Amer. Zool.*, **16**: 220.

Emmel, V. E., 1908. The experimental control of asymmetry at different stages in the development of the lobster. *J. Exp. Zool.*, **5**: 471–484.

Franzini-Armstrong, C., 1970. Natural variability in the length of thin and thick filaments in single fibers from a crab, *Portunus depurator*. *J. Cell Sci.*, **6**: 559–592.

Goudey, L. R., and F. Lang, 1974. Growth of crustacean muscle: asymmetric development of the claw closer muscles in the lobster, *Homarus americanus*. *J. Exp. Zool.*, **189**: 421–427.

Govind, C. K., and F. Lang, 1974. Neuromuscular analysis of closing in the dimorphic claws of the lobster *Homarus americanus*. *J. Exp. Zool.*, **190**: 281–288.

Govind, C. K., J. She, and F. Lang, 1977. Lengthening of lobster muscle fibers by two age-dependent mechanisms. *Experientia*, **33**: in press.

Herrick, F. H., 1896. The American lobster: a study of its habits and development. *Bull. U. S. Fish. Comm.*, **15**: 1–252.

Herrick, F. H., 1911. Natural history of the American lobster. *U. S. Bur. Fish.*, **29**: 149–408.

Hoyle, G., 1973. Correlated physiological and ultrastructural studies on specialized muscles. IIIb. Fine structure of the power-stroke muscle of the swimming leg of *Portunus sanguinolentus*. *J. Exp. Zool.*, **185**: 97–110.

Jahromi, S. S., and H. L. Atwood, 1971a. Structural and contractile properties of lobster leg-muscle fibers. *J. Exp. Zool.*, **176**: 475–486.

Jahromi, S. S., and H. L. Atwood, 1971b. Electrical coupling and growth in lobster muscle fibers. *Can J. Zool.*, **49**: 1029–1034.

Przibram, H., 1931. *Connecting laws in animal morphology.* University of London Press, Ltd., London, 62 pp.

Wiersma, C. A. G., 1955. An analysis of the functional differences between the contractions of the adductor muscles in the thoracic legs of the lobster *Homarus americanus*. *Arch. Neerland. Zool.*, **11**: 1–13.

Reference: *Biol. Bull.*, **152**: 84–104. (February, 1977)

THE LARVAL DEVELOPMENT OF *CLIBANARIUS VITTATUS* (BOSC) (CRUSTACEA: DECAPODA; DIOGENIDAE) REARED IN THE LABORATORY[1]

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Published descriptions of the larval development of members of the Anomuran family Diogenidae are limited to sixteen species in seven of the fifteen genera (see Table I). In addition, Hale (1927) claims a direct development for *Cancellus typus*. *Calcinus verrilli* and six species of *Paguristes* have been reported reared by Provenzano (1968) and *Cancellus spongicola* has been hatched (Provenzano, personal communication), but these descriptions remain unpublished. No larval descriptions exist for species of the genera *Allodardanus, Aniculus, Isocheles, Loxopagurus, Paguropsis, Pseudopagurus,* or *Troglopagurus*. In the genus *Clibanarius* descriptions of the larvae of *C. erythropus* (Hesse, 1876; Issel, 1910; Boraschi, 1921; Bouvier, 1922; Carayon, 1942; Dechancé and Forest, 1958; Pike and Williamson, 1960a, b), an illustration of the larvae of *C. tricolor* (Lewis, 1960), and mention of larvae of unidentified species of *Clibanarius* (Czerniawsky, 1884; Menon, 1937) have been published. Provenzano (personal communication) has reared *C. anomalus*, *C. antillensis*, and *C. tricolor* but none of these descriptions has been published.

Clibanarius vittatus (Bosc) is a very common hermit crab species in intertidal and shallow estuarine water regions of the southeastern coast of the United States. Its known range is from the Potomac River, Gunston, Virginia to Rio de Janeiro, Brazil (Williams, 1965). Although the species has been successfully reared by Provenzano (personal communication) and larval stages have been reared and partially described by Kircher (1967) and Kurata (unpublished), a published description of the larvae of *C. vittatus* has not been completed. The following study was undertaken to provide complete descriptions of laboratory-reared larvae of *C. vittatus* to aid in accurate identification and staging of decapod larvae in field sampling or laboratory investigations.

MATERIALS AND METHODS

On July 7, 1975 numerous specimens of *C. vittatus* were collected by hand from shallow water (temperature, 26° C; salinity 30‰) in the North Inlet estuary, Georgetown, South Carolina. The shells of the hermit crabs were carefully broken and several shell-less ovigerous females were isolated individually in 9 cm Carolina culture dishes containing filtered natural sea water of 25‰ salinity and maintained

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TABLE I

Published descriptions of larvae of Diogenidae.

Genus	Species	Author	Date	Material
Calcinus	*ornatus*	Pike and Williamson	1960b	all stages
	tibicen	Provenzano	1962	all stages
Clibanarius	*erythropus*	Hesse	1876	zoeal stage I only (as *Pagurus misanthropus*)
		Issel	1910	zoeal stage I only (as *C. misanthropus*)
		Boraschi	1921	zoeal stage I only (as *C. misanthropus*)
		*Bouvier	1922	megalopa only (as *Glaucothoe grimaldii*)
		Carayon	1942	megalopa only (as *C. misanthropus*)
		Dechancé and Forest	1958	megalopa only
		Pike and Williamson	1960a	zoeal stage I; megalopa
		Pike and Williamson	1960b	zoeal stages I, II, III
	tricolor	Lewis	1960	unspecified zoeae
Dardanus	*arrosor*	Issel	1910	zoeal stage I only (as *Pagurus arrosor*)
		Boraschi	1921	zoeal stage I only (as *Pagurus arrosor*)
		Bourdillon-Casanova	1960	zoeal stage I only
		Pike and Williamson	1960b	zoeal state I only
		Dechancé	1962	zoeal stage I only
		Kurata	1968a	all stages
	fucosus	Provenzano	1963b	megalopa only (as *Petrochirus diogenes*)
		Biffar and Provenzano	1972	megalopa only
	insignis	Provenzano	1963b	megalopa only
	scutellatus	Provenzano	1963b	megalopa only
	venosus	Provenzano	1963a	megalopa only
Diogenes	*pugilator*	Gurney	1927	all stages
		Menon	1937	all stages
		MacDonald, Pike and Williamson	1957	all stages
		Pike and Williamson	1960a	all stages
		Pike and Williamson	1960b	all stages
	varians (?)	*†Czerniawsky	1884	two zoeal stages only
Paguristes	*digitalis*	Kurata	1968b	all stages
	oculatus	Issel	1910	zoeal stages I and III; megalopa
		Pike and Williamson	1960b	all stages
	sericeus	Rice and Provenzano	1965	all stages
	turgidus	Hart	1937	all stages
Petrochirus	*diogenes*	Provenzano	1968	all stages
Trizopagurus	*magnificus*	Provenzano	1967	all stages

* Not seen by present authors.

† In Russian, extracts in Bouvier (1891).

at 25° C under a 15L:9D light schedule. At 1500 hours on July 8 one brood hatched, and 100 of the most active larvae were placed individually in 6 cm dishes containing 15 ml filtered natural sea water of 25‰ salinity and were maintained

TABLE II

Survival, development time, and duration of the larval stages of Clibanarius vittatus *(Bosc) reared in the laboratory.*

Stage:	Zoea I		Zoea II		Zoea III		Zoea IV		Zoea V		Megalopa	
July hatches:	1975	1976	1975	1976	1975	1976	1975	1976	1975	1976	1975	1976
Survival from first zoeal stage to indicated stage (percentage of original number*)			39	59	25	51	22	48	15	48	11	37
Survival within each stage (percentage of indicated stage to reach subsequent stage)	39	59	64	87	88	98	68	98	73	94	**	33
Time from hatching to reach each stage (days):												
range			8–11	12–17	13–17	19–29	19–24	25–35	25–29	33–43	33–36†	43–51†
x̄			9.4	13.7	15.0	21.8	21.4	29.7	27.5	37.2	35.2	47.0
n			39	46	25	41	21	43	13	33	9	33
Duration of each stage (days):												
range	8–11	12–17	4–7	6–16	6–7	5–10	5–7††	6–9††	5–8	9–11		32–43
x̄	9.4	13.7	5.7	8.3	6.4	7.9	6.3	7.7	7.8	9.9	**	36.3
n	39	46	25	35	21	39	13	34	9	30		3

* 100 for July, 1975; 90 for July, 1976.

** Experiment terminated—megalopae not allowed to molt to first crab.

† Values given for megalopa reached from stage V; for megalopa direct from stage IVb, 30 days ($n = 2$) for 7/75 and 39–47 days ($\bar{x} = 43.5$, $n = 6$) for 7/76.

†† Values given for stage IVa; for stage IVb, 8–9 days ($\bar{x} = 8.5$, $n = 2$) for 7/75 and 9–18 days ($\bar{x} = 13.7$, $n = 6$) for 7/76.

under the conditions described above. Water was changed daily and two drops of concentrated freshly hatched *Artemia* nauplii (San Francisco Bay) were added following each water change.

Individual records were kept for each zoea to determine the number and duration of larval stages. Exuviae from each stage and larvae were preserved in 70% ethyl alcohol. Drawings were made from larvae and exuviae mounted in glycerine using camera lucida. Measurements of preserved larvae were made with an ocular micrometer. Total length (TL) and carapace length (CL) are as described by Pike and Williamson (1960b). Additional larvae were reared during July and August, 1976 to allow for measurements on a larger number of individuals and to check for variations between different broods.

RESULTS

Four or five zoeal stages and a megalopa were obtained through rearing. Rearing in 1975 was terminated before the first crab stage was reached, while in 1976 megalopae were allowed to molt to first crabs. Although similar rearing techniques were used in both instances, the 1976 larvae generally had better survival and longer duration of larval stages. Overall survival values, times to reach each stage, and duration of each stage are summarized in Table II.

Measurements of CL and TL in *C. vittatus* zoeae and megalopa are presented in Table III. Of four hatches of *C. vittatus* measured at stage I, only July, 1975 and July, 1976 were reared. Of particular note is the size variation observed at stage I. Larvae of hatch August, 1975 are consistently larger than those of hatch June, 1976 while July, 1975 and July, 1976 are intermediate in size. As expected with this initial variation, size ranges of later zoeal stages often overlap.

TABLE III

Size ranges and means for carapace length and total length (in mm) of the larval stages of Clibanarius vittatus *(Bosc) reared in the laboratory.*

Stage	Hatch	Carapace Length			Total Length		
		Range	x̄	n	Range	x̄	n
Zoea I	July, 1975	0.9–1.1	1.0	15	1.9–2.2	2.1	15
	August, 1975	1.0–1.1	1.0	10	2.1–2.4	2.3	10
	June, 1976	0.9–1.0	1.0	10	2.0–2.2	2.1	10
	July, 1976	1.0–1.1	1.0	10	2.0–2.3	2.1	10
Zoea II	July, 1975	1.1–1.3	1.2	5	2.3–2.9	2.6	5
	July, 1976	1.1–1.2	1.1	10	2.2–2.7	2.5	10
Zoea III	July, 1975	1.5–1.7	1.6	4	2.8–3.5	3.3	4
	July, 1976	1.7–2.0	1.8	10	3.5–4.1	3.7	10
Zoea IVa	July, 1975	1.7–2.5	2.1	4	3.6–4.4	4.1	4
	July, 1976	2.0–2.5	2.2	10	3.8–5.0	4.4	10
Zoea IVb	July, 1976	2.3–2.6	2.4	4	4.4–5.2	4.8	4
Zoea V	July, 1975	2.6–2.7	2.6	3	4.5–4.8	4.7	3
	July, 1976	2.3–2.7	2.5	9	4.3–5.3	4.9	9
Megalopa	July, 1975	1.1–1.5	1.4	4	3.8–4.1	3.9	4
	July, 1976	1.3–1.4	1.3	3	3.2–3.7	3.4	3

The size of stage IV zoeae molting to megalopa (IVb) is greater than that for stage IV zoeae (IVa) molting to stage V. Size alone is not an adequate or reliable criterion for staging larvae of *C. vittatus.*

Pigmentation of larvae was not studied in detail since the bulk of the material was preserved for later examination and alcohol rapidly bleaches chromatophores in this species. Based on brief observations of freshly sacrificed zoeae, a general pattern is evident. Chromatophores are red or orange-red and present in all stages. Small chromatophores occur ventrally along the rostrum and at the bases of the antenna and antennule. Variable patterns of small chromatophores and a diffuse faint reddish color are found in the mouth parts. At least three distinct chromatophores are found on the ventrolateral carapace surface. The abdomen has a large dorsal chromatophore in each segment and some fine color spots on the ventral surface. A more critical study of pigmentation in *C. vittatus* may provide a useful characteristic for the identification of fresh material.

Descriptions and drawings of each stage follow. The present study has stressed setal numbers; when setal types are mentioned, terminology is based on

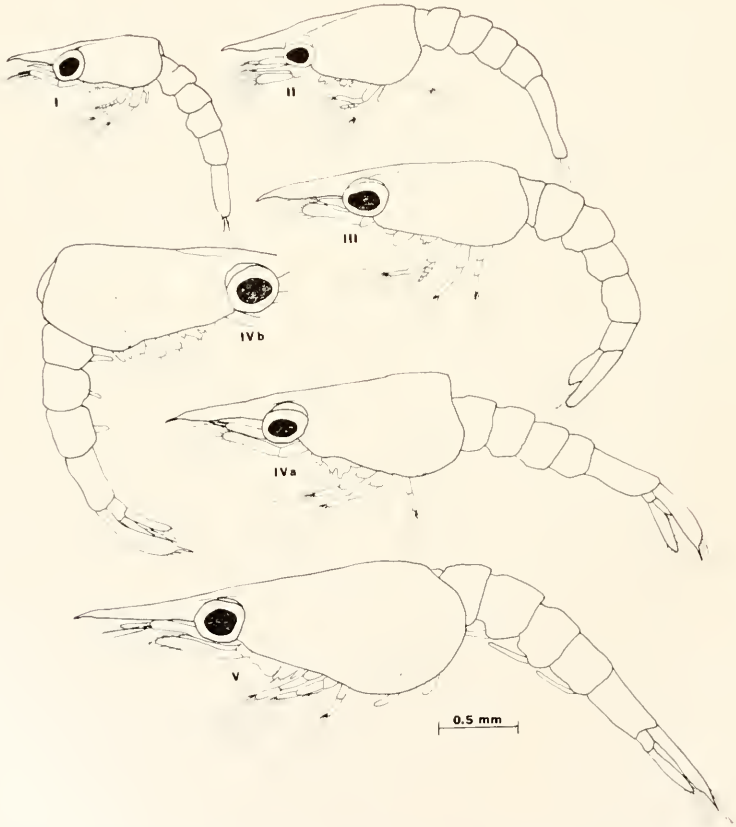


FIGURE 1. *Clibanarius vittatus:* lateral view of zoeal stages I–V. Stage IV zoeae may molt to stage V (IVa) or directly to megalopa (IVb).

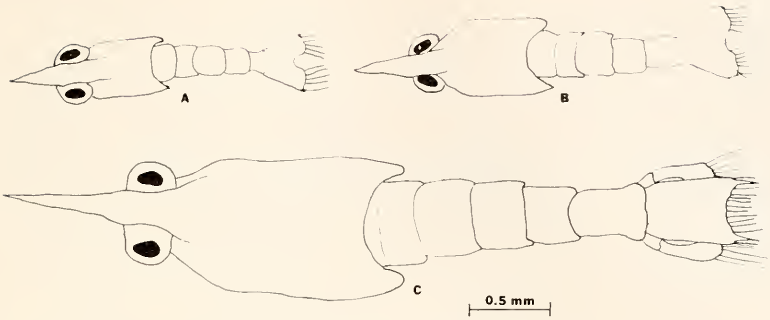


FIGURE 2. *Clibanarius vittatus:* dorsal view of zoeal stages I (A), II (B) and V (C).

that used by Bookhout and Costlow (1974). Abbreviations used are as in Roberts (1970).

Due to the individual variation and complex configuration of the mandibles, only two examples of the configuration and development of the palp are shown (Fig. 6). No attempt has been made to illustrate the mandibles for each stage.

Zoea I (Fig. 1, I, 2A)

The rostrum is long, extending beyond both the antenna and antennule and constricts to a short narrow tip curved ventrad. The carapace is without processes or spines; the postero-lateral border is smoothly rounded. Eyes are sessile. The abdominal somites are plain without spines; the sixth somite and telson are fused. The telson is bilaterally convex with a prominent median notch (Fig. 3A), The telson formula is $7 + 7$; process 1, a very short blunt spine; process 2, a short setose hair; processes 3–7, long plumose setae.

A1 (Fig. 4A): uniramous, unsegmented, with 3 terminal aesthetascs, 2–3 terminal plumose setae, and 1 prominent subterminal seta.

A2 (Fig. 5A): basipod with 1 short spinose spine; endopod about $\frac{2}{3}$ length of scale with 3, rarely 4, terminal setae; scale with 9–11 plumose setae.

Mn (Fig. 6A): large anterior conical tooth and complex array of smaller teeth and projections on biting surfaces; asymmetric, palp absent.

Mx1 (Fig. 7A): coxal endite with 6 setae; basal endite with 1 denticulate and 2 denticulate cuspidate setae and occasionally 1 short simple seta; endopod with no distinct segmentation, 3 plumose setae.

Mx2 (Fig. 8A): coxal and basal endites bilobed; coxal endite with 6 setae on proximal lobe, 4 setae on distal lobe; basal endite with 5 setae on proximal lobe, 3–4 setae on distal lobe; endopod with 2 terminal and 2 subterminal setae, fringed edges; scaphognathite with 4–6 plumose setae.

Mxp1 (Fig. 9A): basis with 7–9 fine setae on inner margin; endopod 5-segmented, fringed on outer margin of segments 2–4, 1 plumose seta at distal margin of 4th segment 2, 2, 1, 2 fine setae on inner margin of segments 1–4; segment 5 with 3 denticulate setae and 1 simple seta terminal. Exopod with 4 terminal natatory plumose setae.

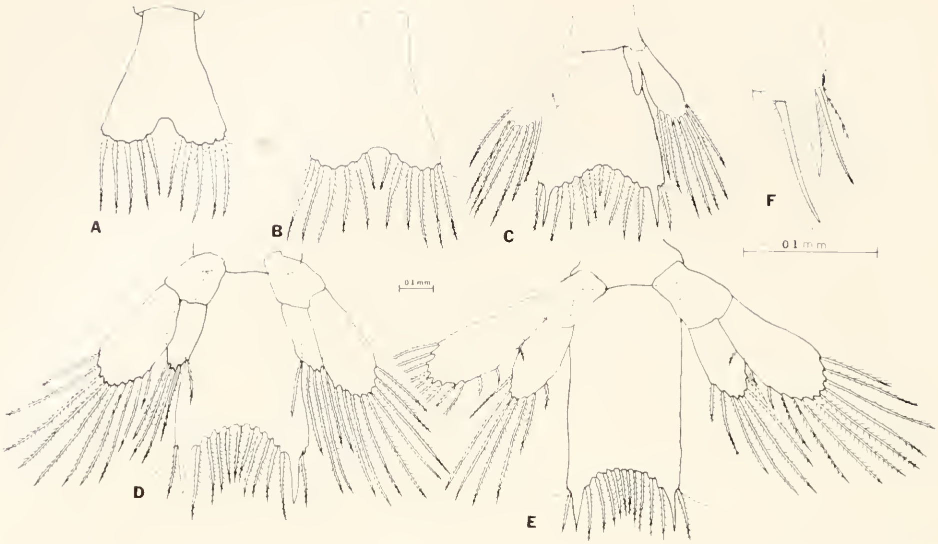


FIGURE 3. *Clibanarius vittatus:* telson of zoeal stage I (A), II (B), III (C), IV (D), and V (E). Detail of telson processes 1–5 (F) indicates pattern found in stages III–V.

Mxp2 (Fig. 10A): basis with 3 fine setae on inner margin; endopod 4-segmented, 2 fine setae each on inner margin of segments 1–3, segment 4 with terminal plumose or denticulate setae, 1 plumose seta on outer margin; exopod with 4 terminal natatory plumose setae.

Mxp3 (Fig. 10a): uniramous exopod without setae.

Zoea II (Fig. 1, II, 2B)

Except for size increase, the rostrum and carapace are as in stage I. The eyes are stalked. The telson has a shallow notch medially with convex lobes (Fig. 3B). The telson formula is 8 + 8; process 1, greatly reduced or absent (Fig. 3F); process 2, a short plumose hair; processes 3–8, plumose setae. Pleopod buds and uropods are absent.

A1 (Fig. 4B): uniramous, 2-segmented; peduncle with 3–4 long plumose setae and 0–2 short setae at base of distal segment; distal segment with 2 terminal aesthetascs, 2–3 terminal fine plumose and 2 subterminal aesthetascs.

A2 (Fig. 5B): basipod unchanged; endopod with 4 setae; scale with 11 plumose setae.

Mn: increase in size and number of smaller teeth.

Mx1 (Fig. 7B): coxal endite with 6 setae; basal endite with 4 denticulate cuspidate setae, 1 short plumose seta and 1 very short simple seta; endopod with 3–4 plumose setae.

Mx2 (Fig. 8B): coxal endite with 6–7 setae on proximal lobe, 3–4 setae on distal lobe; basal endite with 4–5 setae on proximal lobe, 3–4 setae on distal lobe; endopod unchanged; scaphognathite with 7 plumose setae.

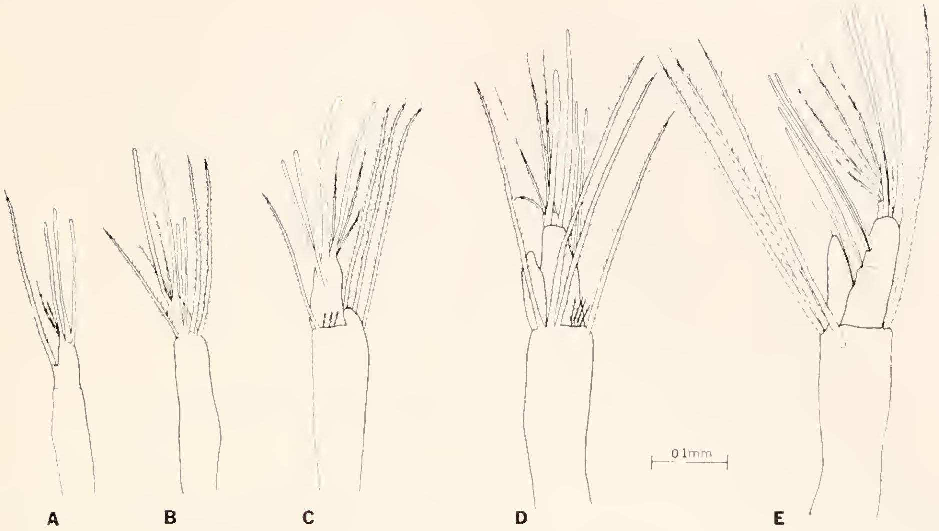


FIGURE 4. *Clibanarius vittatus:* antennule of zoeal stage I (A), II (B), III (C), IV (D), and V (E).

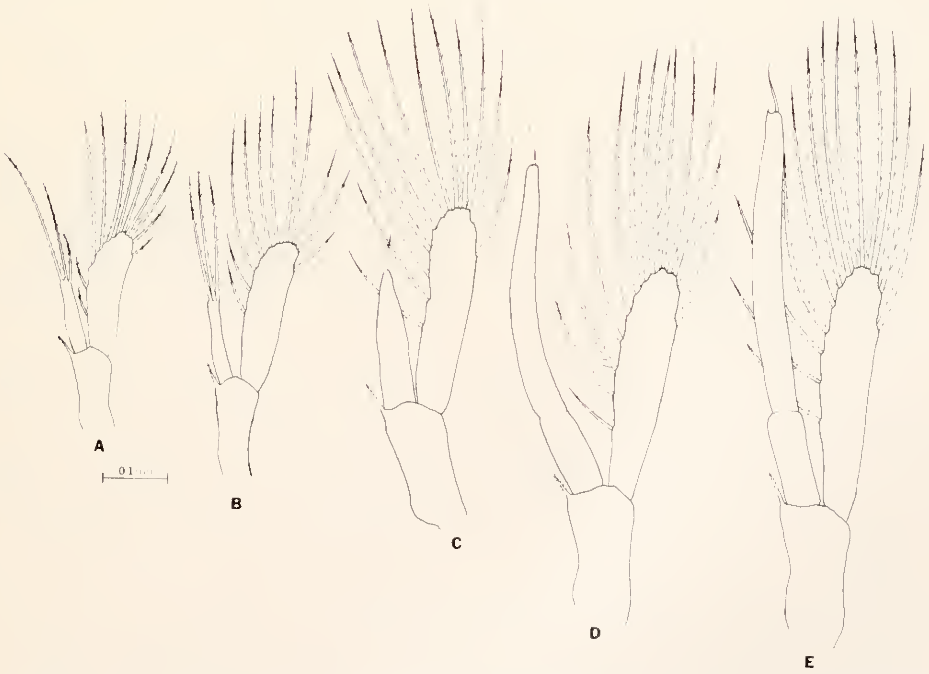


FIGURE 5. *Clibanarius vittatus:* antenna of zoeal stage I (A), II (B), III (C), IV (D), V (E).

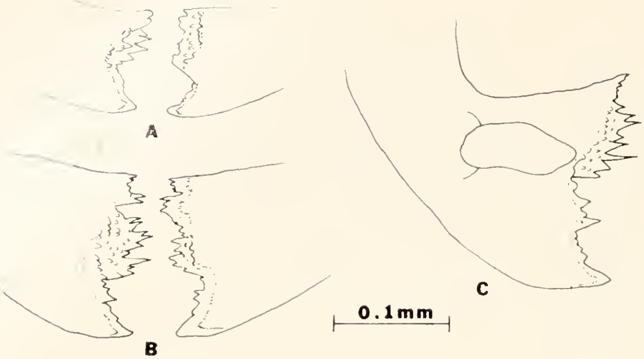


FIGURE 6. *Clibanarius vittatus:* mandibles of zoeal stage I (A), III (B) and anterior view of zoeal stage V (C).

Mxpl (Fig. 9B): basis unchanged; endopod 5-segmented, 1 seta on outer margin of each segment 1–4, 2, 2, 1, 2 fine setae on inner margin of segments 1–4, segment 5 with 3 denticulate setae and 1 simple or denticulate seta terminal; exopod with 6 terminal natatory plumose setae.

Mxp2 (Fig. 10B): basis with 4 fine setae on inner margin; endopod 4-segmented, 2 fine setae on inner margin of each segment 1–3, 0, 1, 1, 1 setae on outer margin of segments 1–4, segment 4 with 4 terminal serrate or denticulate setae; exopod with 6 terminal natatory plumose setae.

Mxp3 (Fig. 10b): uniramous with 5, sometimes 4, terminal natatory setae.

Zoea III (Fig. 1, III)

The rostrum and carapace remain unchanged. An articulation between the sixth abdominal somite and the telson first appears. Uropods (U) present with exopod and endopod fused. Pleopod buds are usually absent or very rudimentary. The posterior margin of the telson is concave with no notch or bilateral lobes (Fig. 3C). The telson formula is 8 + 1 + 8; process 1, a small stub, process 2, a plumose hair; process 3 a plumose seta; process 4, a fixed spine; processes 5–8 and medial process, plumose setae.

A1 (Fig. 4C): uniramous, 2-segmented; peduncle with 4 long plumose setae and 3–4 short setae at base of distal segment; distal segment with 2 thick terminal aesthetascs, 3 terminal fine plumose setae, and 2 fine subterminal aesthetascs.

A2 (Fig. 5C): basipod unchanged; endopod with increased diameter, setae reduced in number to 1 simple terminal process; scale with 11–12 plumose setae.

Mn (Fig. 6B): essentially same as earlier stages with increased complexity of denticulation.

Mx1 (Fig. 7C): coxal endite with 6–7 setae; basal endite setae larger than stage II but number unchanged; endopod unchanged.

Mx2 (Fig. 8C): coxal endite proximal lobe may increase to 8 setae; scaphognathite with 7–10 plumose setae; other setal counts as in stage II.

Mxp1 (Fig. 9C): setation as in stage II.

Mxp2 (Fig. 10C): setation as in stage II.

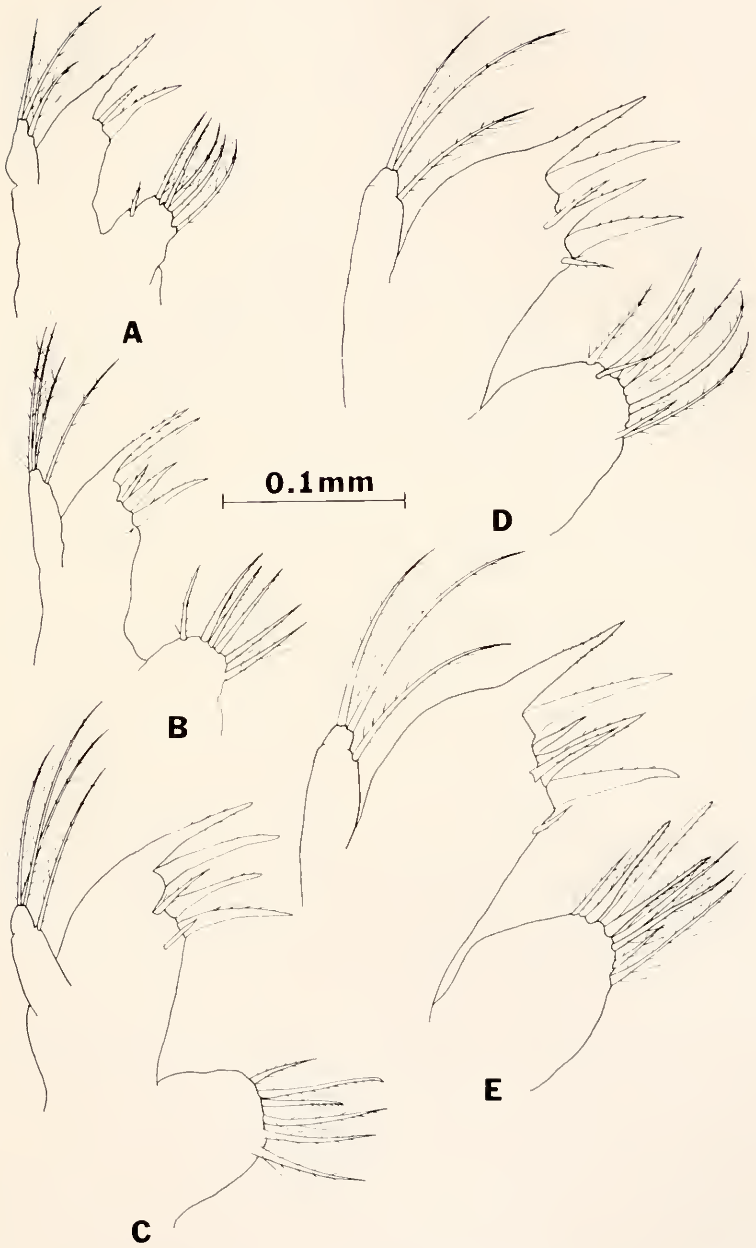


FIGURE 7. *Clibanarius vittatus:* maxillule of zoeal stage I (A), II (B), III (C), IV (D), V (E).

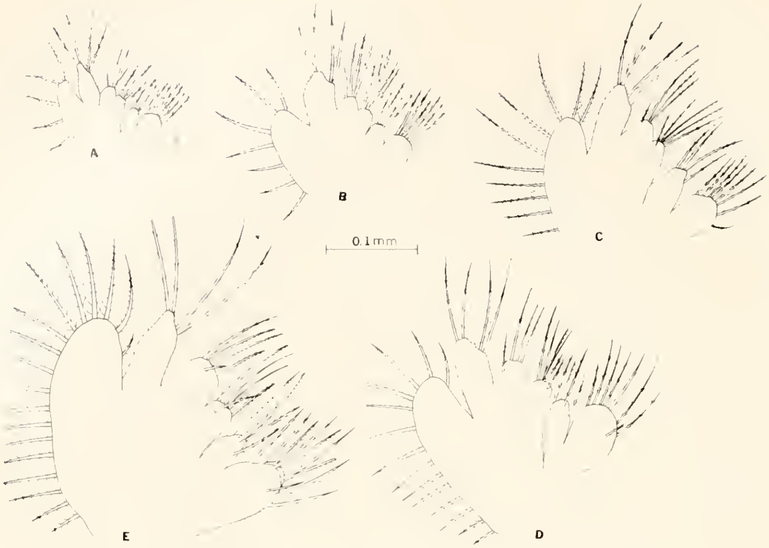


FIGURE 8. *Clibanarius vittatus:* maxilla of zoeal stage I (A), II (B), III (C), IV (D), V (E).

Mxp3 (Fig. 10c): uniramous 3-segmented with 5–6 terminal natatory plumose setae.

U (Fig. 3C): exopod fused to propod, 6–9 plumose setae present; endopod simple bud.

Zoea IV (Fig. 1, IV, a b)

The rostrum and carapace remain unchanged. Pleopods (P1) vary from not present (Fig. 1, IV a) to conspicuous buds (Fig. 1, IV b). Leg buds may be present. The uropod has an articulated exopod and endopod. The telson formula may be the same as stage III or increase by 1 seta to 9 + 9 (Fig. 3D). Larger individuals with well-developed leg and pleopod buds will molt directly into the megalopa.

A1 (Fig. 4D): biramous, 2-segmented; peduncle unchanged; inner ramus, plain bud up to $\frac{2}{3}$ length of outer ramus; outer ramus with 2–3 terminal aesthetascs, 4 terminal setae and 2 or more fine subterminal aesthetascs.

A2 (Fig. 5D): basipod unchanged; endopod longer than scale, weakly segmented with terminal process; scale with 11–14 plumose setae.

Mn: palp bud may be evident.

Mx1 (Fig. 7D): coxal endite with 7–8 setae; remaining setation as in stage III.

Mx2 (Fig. 8D): proximal lobes of coxal and basal endites may increase to 9 and 6 setae, respectively; scaphognathite with 12–14 plumose setae.

Mxp1 (Fig. 9D): setation as in stage II.

Mxp2 (Fig. 10D): endopod setation as in stage II; exopod with 6–7 terminal natatory plumose setae.

Mxp3 (Fig. 10d): setation as in stage III.

$P1_2$ to $P1_5$: small uniramous buds variable in length.

U (Fig. 3D): exopod and endopod articulated; exopod with 8–12 plumose setae, endopod with 3–6 setae.

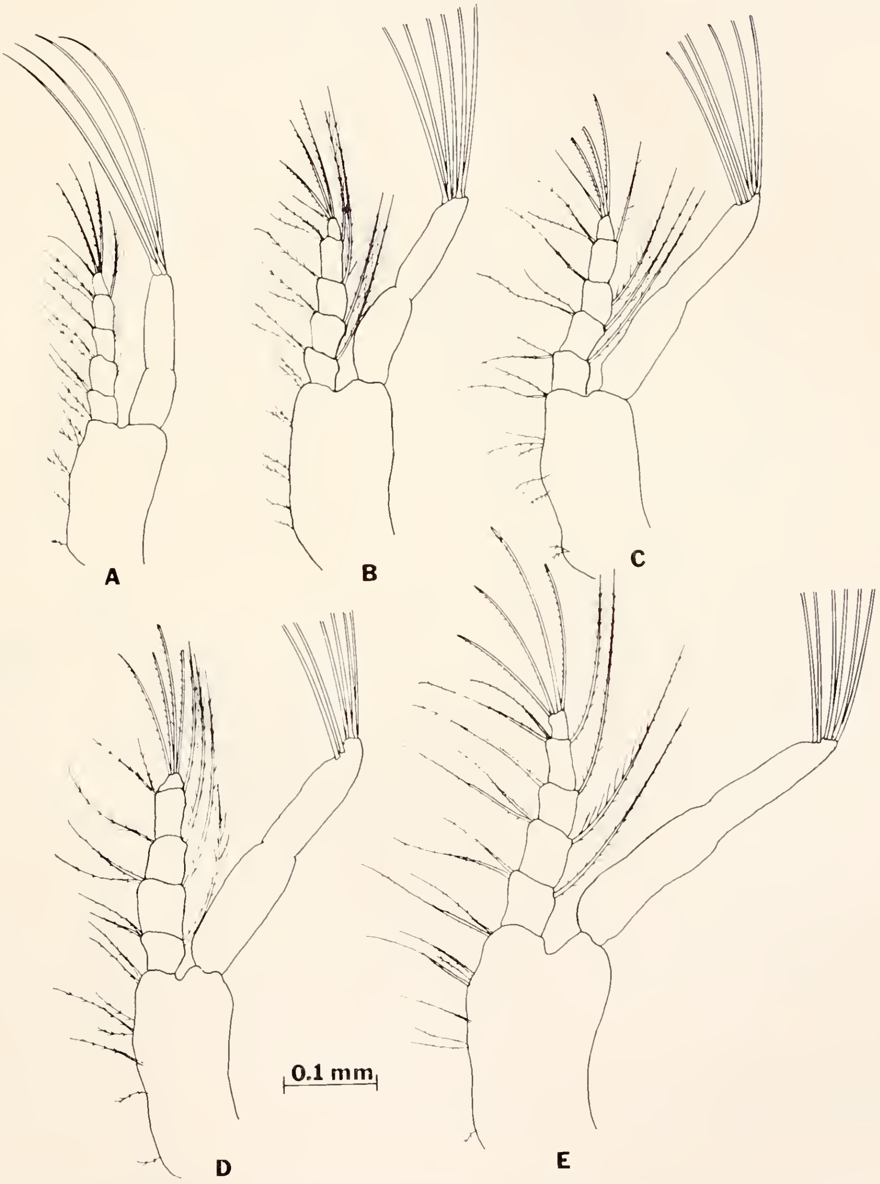


FIGURE 9. *Clibanarius vittatus:* first maxilliped of zoeal stage I (A), II (B), III (C), IV (D), V (E).

Zoea V (*Fig. 1, V, 2C*)

The postero-lateral carapace is somewhat expanded. Pleopod and leg buds are always well-developed and at times exhibit segmentation and some setae (pleopods with 3–4 terminal setae and leg buds with bristles). The telson is nearly rectangular with a concave posterior border (Fig. 3E). The telson formula is 9 + 9 with process types as outlined for stage III.

A1 (Fig. 4E): biramous, 2-segmented; peduncle unchanged; inner ramus

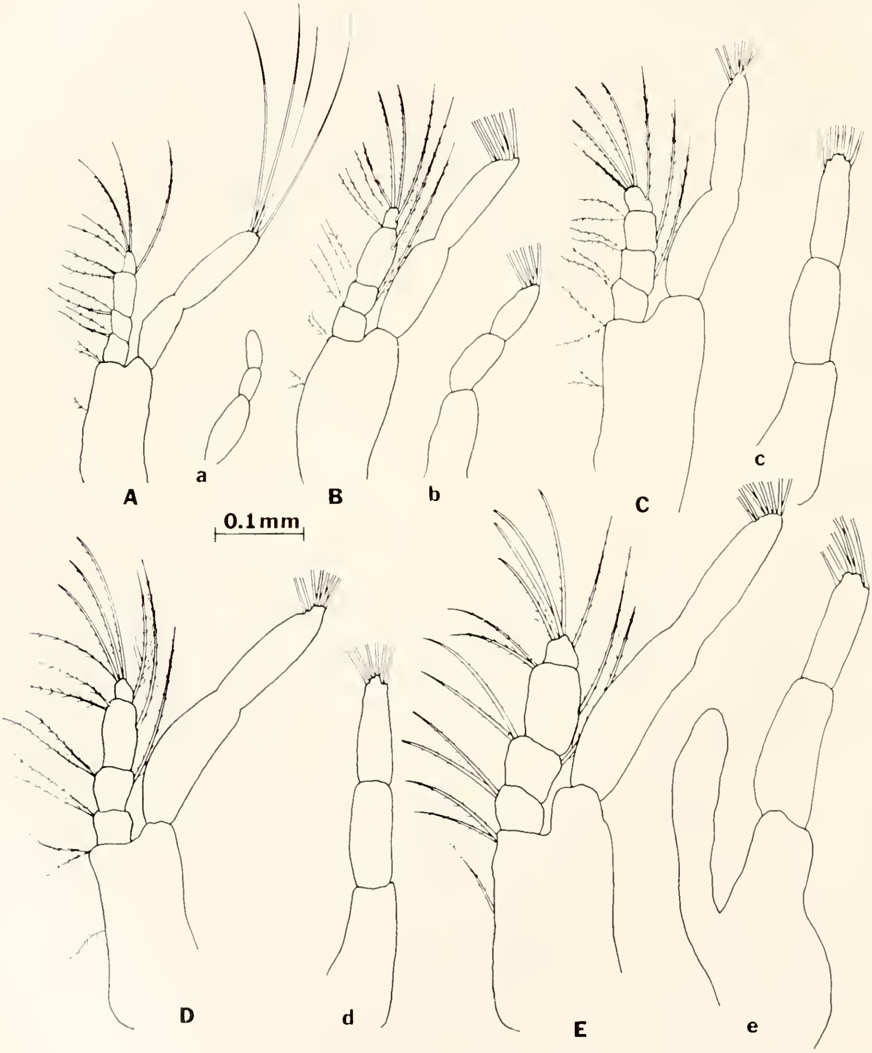


FIGURE 10. *Clibanarius vittatus:* second (A-E) and third (a-e) maxillipeds of zoeal stage I (A, a), II (B, b), III (C, c), IV (D, d), V (E, e).

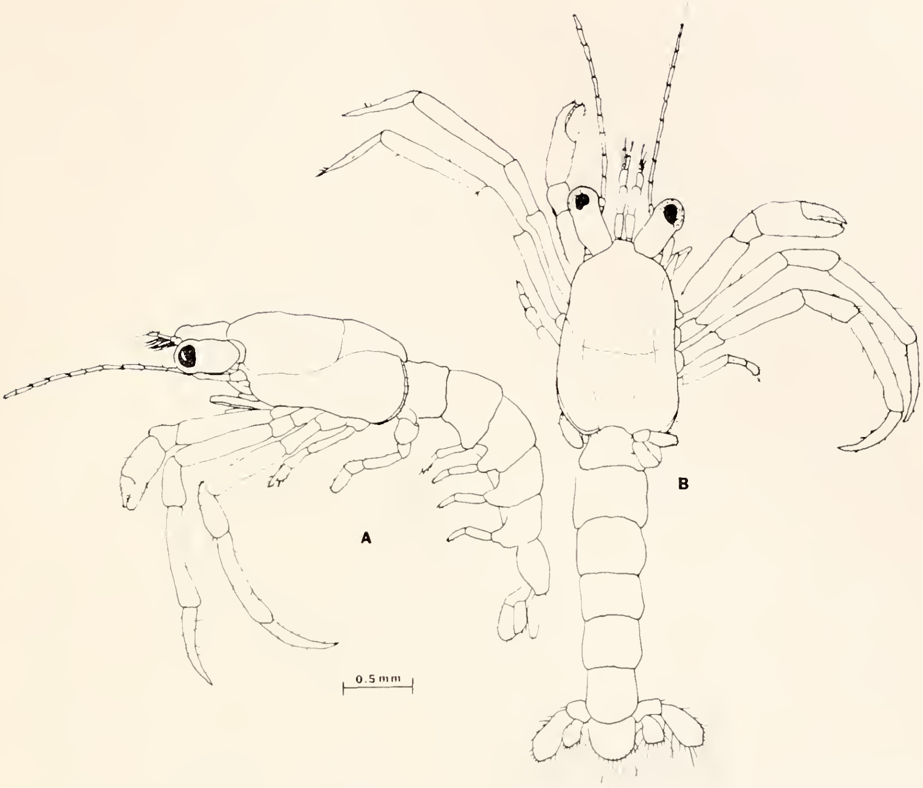


FIGURE 11. *Clibanarius vittatus:* dorsal (A) and lateral (B) views of megalopa.

may have a fine terminal hair; outer ramus with 3 terminal aesthetascs, 4 terminal setae and 4 or more subterminal aesthetascs.

A2 (Fig. 5E): basipod unchanged; endopod longer than scale, 2-segmented with terminal process; scale with 13–14 plumose setae.

Mn (Fig. 6C): simple palp present.

Mx1 (Fig. 7E): coxal endite with 8 setae; basal endite may have 1 additional small seta relative to stage IV; no change in endopod.

Mx2 (Fig. 8E): coxal endite with 9–10 setae on proximal lobe, 4 setae on distal lobe; basal endite with 5–6 setae on proximal lobe, 4–5 setae on distal lobe; endopod unchanged; scaphognathite with 15–19 plumose setae.

Mxp1 (Fig. 9E): setation as in stage II.

Mxp2 (Fig. 10E): endopod as in stage II; exopod with 8 terminal natatory plumose setae.

Mxp3 (Fig. 10e): endopod bud evident on basis; exopod with 6 terminal natatory plumose setae.

Pl_2 to Pl_5: long uniramous buds, at times with 3–4 terminal setae.

U (Fig. 3E): exopod and endopod articulated; exopod with 9–12 plumose setae, endopod with 5–7 plumose setae.

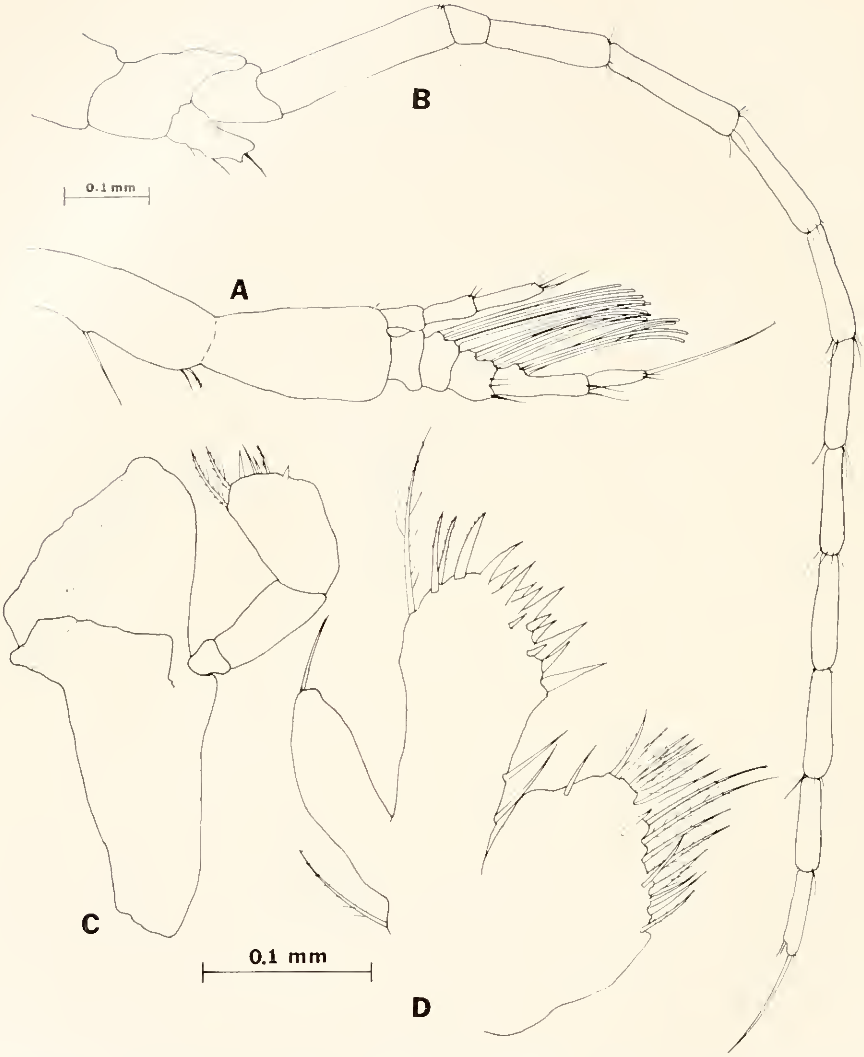


Figure 12. *Clibanarius vittatus;* megalopa appendages: antenna (A), antennule (B), mandible—dorsal view (C), and maxillule (D).

Megalopa (Fig. 11)

The carapace is shorter than the abdomen; the rostrum is reduced to a small blunt projection. The posterolateral carapace margin is fringed with simple setae; no spines are present. The telson (Fig. 14E) is symmetrical, the posterior margin convex with 8–9 long plumose setae and a variable number of short processes on the margin and dorsal surface. The total length of the eye and stalk is about twice the width. The cornea is slightly enlarged. No ocular scales are present.

A1 (Fig. 12A): outer ramus 5-segmented with 4 aesthetascs on segment 2, 4 aesthetascs and a few fine simple setae on segment 3, 3 aesthetascs and 2 simple setae on segment 4, and 2 subterminal simple setae and 1 long terminal simple seta on segment 5; inner ramus 3-segmented with 2 simple setae on segment 2 and 5–6 simple setae on segment 3.

A2 (Fig. 12B): flagellum with 11 segments, all with short setae distally and 1 longer seta on terminal segment tip; scale reduced with about 3 simple setae.

Mn (Fig. 12C): cuplike with prominent smooth ventral edge and short raised dorsal edge; palp 3-segmented with 2 short serrate setae and 5–7 short simple setae on terminal segment.

Mx1 (Fig. 12D): coxal endite with about 18–20 setae; basal endite with 2 subterminal setae, 16 terminal cuspidate and denticulate setae and 1 thick plumose seta; endopod unsegmented with 1 terminal simple seta and no palp; one plumose seta present on inner margin of basis.

Mx2 (Fig. 13A): coxal and basal endites bilobed with numerous setae; endopod unsegmented without setae; scaphognathite with about 60 plumose setae.

Mxp1 (Fig. 13B): bilobed basis, the proximal lobe with 5 setae, the distal lobe with about 20 setae; endopod unsegmented with 8–10 plumose setae along outer margin.

Mxp2 (Fig. 13C): basis with 3 plumose setae on inner margin, endopod 4-segmented with 4 denticulate setae on segment 3 and 6 terminal spines on segment 4; exopod with 6–8 terminal plumose setae.

Mxp3 (Fig. 13D): basis with 2–3 plumose setae on inner margin; endopod 5-segmented with complex setation; exopod with 6 long plumose setae and 2–4 short plumose setae.

P_1 to P_5 (Fig. 11, 14): chelipeds equal with setae most numerous on claws; P_1 distinctly shorter than P_2 and P_3; P_2 and P_3 similar, with dactyl about $\frac{2}{3}$ length of propodus; P_4 and P_5 shorter and subchelate with tubercles and numerous simple setae on terminal segments (Fig. 14B, C).

Pl_2 to Pl_5 (Fig. 14D): paired on abdominal segments 2–5; biramous, inner ramus, simple unarmed lobe, outer ramus with 8–9 plumose setae.

U (Fig. 14E): equal in size; propod with simple setae at outer margin of exopod articulation; inner margin forms raised edge at endopod articulation but no spine present; exopod with 12–20 plumose setae and about 6–7 tubercles; endopod with 8–15 plumose setae.

DISCUSSION

The constricted tip of the rostrum and lack of spines on the carapace and abdominal somites in all stages and the deeply notched telson of stage I readily distinguish the zoeae of *C. vittatus* from pagurid larvae in South Carolina waters. However, these features are also shared by the zoeae of *C. erythropus* (Pike and Williamson, 1960b), *C. anomalus*, *C. antillensis*, *C. tricolor* (Provenzano, personal communication) and *C. zebra* (Hazlett, personal communication). Therefore, in regions where more than one species of *Clibanarius* are present, additional features will be required to separate species.

Staging of zoeae I–IV for *C. vittatus* may be accomplished using maxilliped setation and telson/uropod characters as outlined by Pike and Williamson (1960b). The separation of stage IV and V is more difficult since the maxillipeds, telsons,

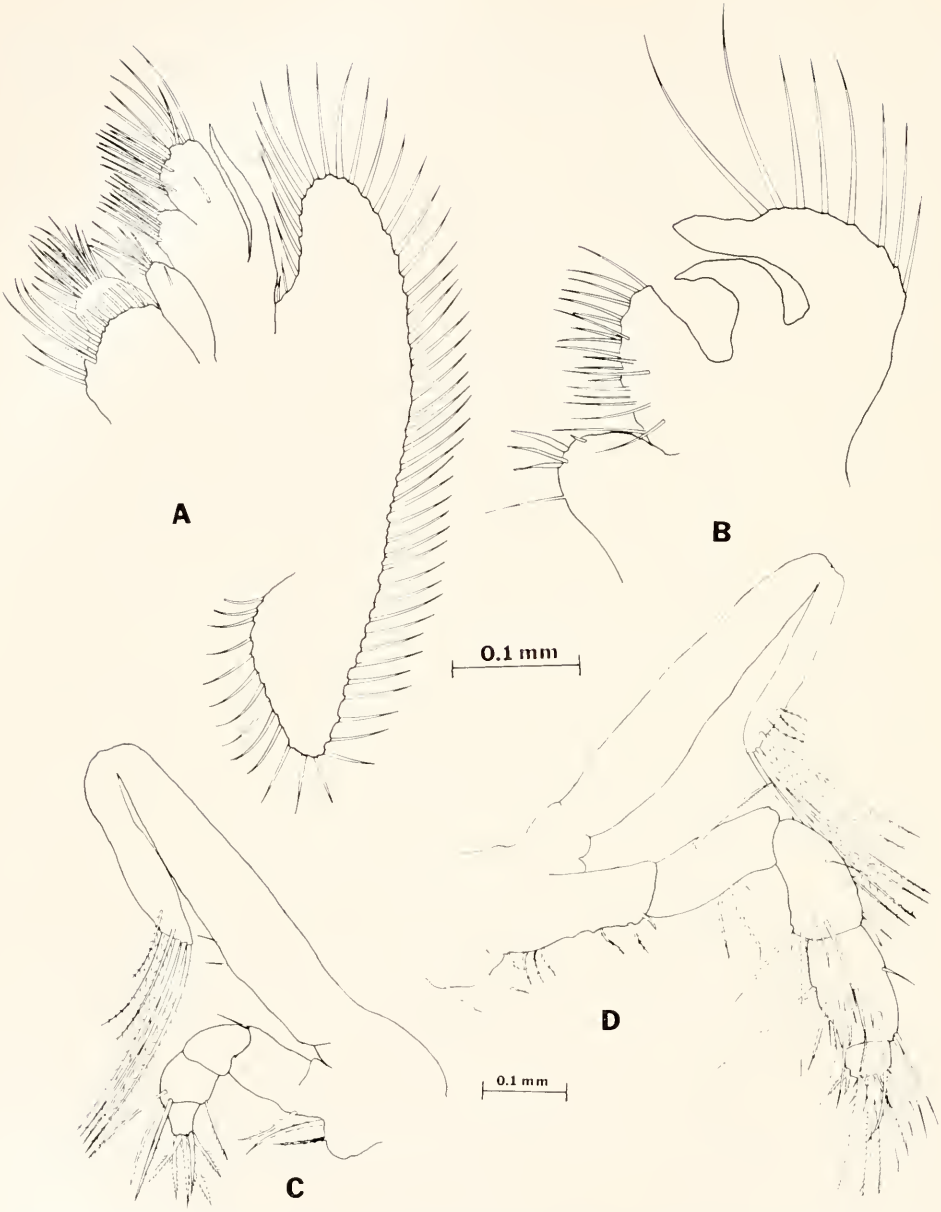
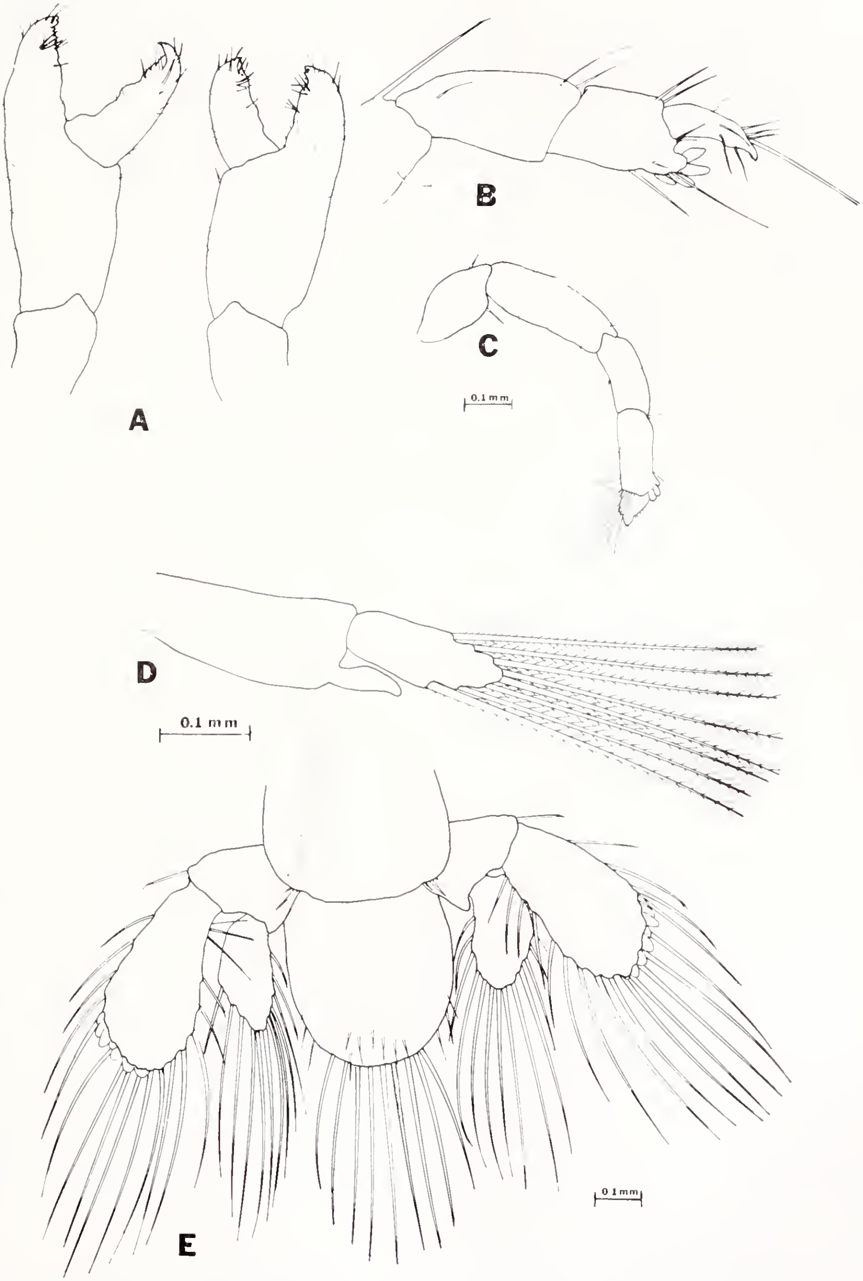


Figure 13. *Clibanarius vittatus*, megalopa appendages: maxilla (A), first maxilliped (B), second maxilliped (C), and third maxilliped (D).

and uropods are nearly identical in the two stages. Pike and Williamson (1960b) note the presence of pleopods in stage V diogenid larvae as opposed to their absence in stage IV. In *C. vittatus*, pleopods may be present in both stages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B
C
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D
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E
0.1 mm

FIGURE. 14. *Clibanarius vittatus*, megalopa appendages: right and left chelipeds (A) fourth pereiopod (C), fourth pleopod (D), and telson (E).

although less common in stage IV. The most reliable single characteristic to separate stages IV and V in *C. vittatus* is the lack of mandibular palps in stage IV.

The most common developmental series for *C. vittatus* is five zoeal stages. A short series of four zoeal stages was observed eight times in the present study and also noted by Kircher (1967). The number of zoeal stages for the Diogenidae is variable, ranging from two zoeae in *Paguristes sericeus* (Rice and Provenzano, 1965) to 6–8 zoeae in *Calcinus tibicen* (Provenzano, 1962).

Available characteristics of diogenid larvae have been reviewed by Provenzano (1968). The constricted rostrum and lack of abdominal spines may prove to be characteristic for the genus *Clibanarius,* but larvae of only two species have been described in detail to date. In *C. erythropus* (Pike and Williamson, 1960b) the telson and sixth abdominal somite are first articulate in stage II, as opposed to stage III in *C. vittatus.* In stage III larvae the fourth telson process is reduced to a small tubercle in *C. erythropus,* while the process is retained as a prominent fused spine in *C. vittatus.* Ranges of setation counts also vary in some appendages, but, in general, the zoeae of the two species are very similar.

The megalopa of *C. vittatus* may be recognized in plankton samples from South Carolina by its equal-sized chelipeds and suboval telson with nine long marginal setae. The second and third pereiopods are nearly identical. Distinctive spines or teeth on the dactyl or propodus, as seen in certain other diogenid larvae (Provenzano, 1968), are absent.

As the only other megalopa described for the genus is that of *C. erythropus* (Dechancé and Forest, 1958), a detailed discussion of generic and specific characteristics is impossible at present. However, based on key morphological characteristics of the megalopa listed by Provenzano (1968), certain points should be noted. The ventral antennular ramus is three-segmented in *C. vittatus, C. erythropus,* and *C. tricolor* (Provenzano, 1968). The slightly dilated cornea, lack of eye scales, carapace shape, uropods, and telson are all similar in *C. vittatus* and *C. erythropus.* The flagellum of the antenna consists of eleven segments in *C. vittatus,* while there are only seven segments in *C. erythropus* and *C. tricolor.* The endopod tip in the megalopa of *C. erythropus* is distinctly recurved (Dechancé and Forest, 1958); in the megalopa of *C. vittatus* it is straight and very similar to that described for *Petrochirus diogenes* (Provenzano, 1968).

Despite unexplained differences in stage duration and mortality, the morphology of the two hatches of *C. vittatus* larvae reared in this laboratory varied little from each other. Major morphological features were consistant while setal counts varied within similar ranges. Plankton tows from North Inlet yielded only stage I zoeae, making planned comparisons with "wild" larvae impossible. In general, descriptions of laboratory reared decapod larvae appear adequate to identify and usually stage "wild" larvae but detailed comparisons are rare and should be pursued.

We wish to express our appreciation to Dr. Anthony J. Provenzano, Jr., Old Dominion University, Norfolk, Virginia, for his review of this manuscript, and to Dr. Brain A. Hazlett, University of Michigan, Ann Arbor, Michigan, for providing figures of *C. zebra* larvae. This research was supported, in part, by EPA grant #R804407010.

SUMMARY

1. A table of previous descriptions of larvae of Diogenidae is given.

2. Larvae of the hermit crab *Clibanarius vittatus* were reared on a diet of *Artemia* nauplii. Five, rarely four, zoeal stages and a megalopa were obtained. Duration, mortality and sizes of larvae from two hatches are listed.

3. Detailed descriptions and figures for each larval stage are presented.

LITERATURE CITED

BIFFAR, T. A., AND A. J. PROVENZANO, JR., 1972. A re-examination of *Dardanus venosus* (H. Milne Edwards) and *D. imperator* (Miers), with a description of a new species of *Dardanus* from the western Atlantic (Crustacea, Decapoda, Diogenidae). *Bull. Mar. Sci.,* **22**: 777–805.

BOOKHOUT, C. G., AND J. D. COSTLOW, JR., 1974. Larval development of *Portunus spinicarpus* reared in the laboratory. *Bull. Mar. Sci.,* **24**: 20–51.

BORASCHI, L., 1921. Osservazioni sulle larvae dei crostacei decapodi: brachiuri e anomuri. *Mem. Reale Com. Talassogr. Ital.,* **87**: 1–32.

BOURDILLON-CASANOVA, L., 1960. Le meroplancton du Golfe de Marseille: les larves de Crustacés Décapodes. *Rec. Trav. Sta. Mar. Endoume Fac. Sci. Marseille,* **18**: 1–286.

BOUVIER, E. L., 1891. Les Glaucothoés, sont-elles des larves de Pagures? *Ann. Sci. Nat. Zool.,* **12**: 65–82.

BOUVIER, E. L., 1922. Observations complémentaires sur les Crustacés Décapodes provenant des campagnes de S. A. S. le Prince de Monaco. Pages 1–106 in *Resultats des campagnes scientifique accomplies sur son yacht par Albert I, publies sous sa direction avec le concours de M. Jules Richard.* Monaco, Fasc. 62.

CARAYON, J., 1942. Sur le stade glaucothoe du pagure *Clibanarius misanthropus* et sur l'etablissement de la dissymetrie chez les pagures. Comparison avec *Glaucothoe grimaldii. C. R. Hebd. Séanc. Acad. Sci. Paris,* **214**: 387–389.

CZERNIAWSKY, V., 1884. Crustacea Decapoda pontica littoralia. *Schr. Nat. Gesell. Charkoff,* **13**: 1–268.

DECHANCÉ, M., 1962. Remarques sur les premiers stades larvaires de plusiers espèces indo-pacifiques du genre *Dardanus* (Crustacés Décapodes Pagurides). *Bull. Mus. Nat. Hist. Natur. Paris,* **34**: 82–94.

DECHANCÉ, M., AND J. FOREST, 1958. Les glaucothoés de *Catapaguroides timidus* (Roux) et de *Clibanarius erythropus* (Latreille). Remarques sur le stade post-larvaire des pagurides. *Bull. Soc. Zool. Fr.,* **83**: 274–293.

GURNEY, R., 1927. Report on the larvae of the Crustacea Decapoda. Zoological results of the Cambridge Expedition to the Suez Canal. *Trans. Zool. Soc. London,* **22**: 231–286.

HALE, H. M., 1927. *The crustaceans of South Australia. Part I. (Handbooks of the flora and fauna of South Australia).* Harrison Weir, Adelaide, 201 pp.

HART, J. F. L., 1937. Larval and adult stages of British Columbia Anomura. *Can. J. Res.,* **D15**: 179–220.

HESSE, E., 1876. Description des Crustacés rares ou nouveaux des côtes de France. *Ann. Sci. Nat. Zool.,* **5**: 1–42.

ISSEL, R., 1910. Ricerche intorno alla biologia ed alla morfologia dei crostacei decapodi. Parte I. Studi sui Paguridi. *Arch. Zool. Ital.,* **4**: 335–397.

KIRCHER, A. B., 1967. The larval development of *Clibanarius vittatus* and *Hypoconcha arcuata* in six salinities. *Masters thesis, Duke University,* Durham, North Carolina, 143 pp.

KURATA, H., 1968a. Larvae of Decapoda Anomura of Arasaki, Sagami Bay—II. *Dardanus arrosor* (Herbst) (Diogenidae). *Bull. Tokai. Reg. Fish. Res. Lab.,* **56**: 173–180.

KURATA, H., 1968b. Larvae of Decapoda Anomura of Arasaki, Sagami Bay—III. *Paguristes digitalis* (Stimpson) (Diogenidae). *Bull. Tokai. Reg. Fish. Res. Lab.,* **56**: 181–186.

LEWIS, J. B., 1960. The fauna of rocky shores of Barbados, West Indies. *Can. J. Zool.,* **38**: 391–435.

MACDONALD, J. D., R. B. PIKE, AND D. I. WILLIAMSON, 1957. Larvae of the British species of *Diogenes, Pagurus, Anapagurus* and *Lithodes* (Crustacea, Decapoda). *Trans. Zool. Soc. London,* **128**: 209–257.

MENON, M. K., 1937. Decapod larvae from the Madras plankton. *Bull. Madras Govt. Mus. (Natur. Hist. Sect.)*, **3**: 1–56.

PIKE, R. B., AND D. I. WILLIAMSON, 1960a. Crustacea, Decapoda: larvae. XI. Paguridea, Coenobitidea, Dromiidea and Homolidae. *Fich. Ident. Zooplankton*, **81**: 1–9.

PIKE, R. B., AND D. I. WILLIAMSON, 1960b. Larvae of decapod Crustacea of the families Diogenidae and Paguridae from the Bay of Naples. *Publ. Sta. Zool. Napoli*, **31**: 493–552.

PROVENZANO, A. J., JR., 1962. The larval development of *Calcinus tibicen* (Herbst) (Crustacea, Anomura) in the laboratory. *Biol. Bull.*, **123**: 179–202.

PROVENZANO, A. J., JR., 1963a. The glaucothoë stage of *Dardanus venosus* (H. Milne-Edwards) (Decapoda: Anomura). *Bull. Mar. Sci. Gulf Caribbean*, **13**: 11–22.

PROVENZANO, A. J., JR., 1963b. The glaucothoes of *Petrochirus diogenes* (L.) and two species of *Dardanus* (Decapoda: Diogenidae). *Bull. Mar. Sci. Gulf Caribbean*, **13**: 242–261.

PROVENZANO, A. J., JR., 1967. The zoeal stages and glaucothoe of the tropical Eastern Pacific hermit crab, *Trizopagurus magnificus* (Bouvier, 1898) (Decapoda: Diogenidae), reared in the laboratory. *Pac. Sci.*, **21**: 457–473.

PROVENZANO, A. J., JR., 1968. The complete larval development of the West Indian hermit crab *Petrochirus diogenes* (L.) (Decapoda, Diogenidae) reared in the laboratory. *Bull. Mar. Sci.*, **18**: 143–181.

RICE, A. L., AND A. J. PROVENZANO, JR., 1965. The zoeal stages and the glaucothoe of *Paguristes sericeus* A. Milne-Edwards (Anomura, Diogenidae). *Crustaceana*, **8**: 239–254.

ROBERTS, M. H., JR., 1970. Larval development of *Pagurus longicarpus* Say reared in the laboratory, I. Description of larval instars. *Biol. Bull.*, **139**: 188–202.

WILLIAMS, A. B., 1965. Marine decapod crustaceans of the Carolinas. *Fish. Bull. Fish Wildlife Serv. U. S.*, **65**: 1–298.

Reference: Biol. Bull., **152**: 105-119. (February, 1977)

CYTOCHEMICAL ASPECTS OF *MERCENARIA MERCENARIA* HEMOCYTES

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Much work has been published on the cytology of molluscan hemocytes. Cuénot (1891), working with several lamellibranchs, recognized the existence of three cell types; Drew and Cantab (1910) also classified hemocytes of *Cardium norvegicum* into three morphological classes. Takatsuki (1934) described two hemocyte types in *Ostrea edulis.*

Zacks and Welch (1953) demonstrated cholinesterase and lipase in hemocytes of *Mercenaria mercenaria,* and Zacks (1955) published the first extensive cytological and cytochemical study on the blood cells of this animal. Only one type of cell was described by Zacks which he termed a granulocyte. Feng (1965) demonstrated that hemocytes of *Crassostrea virginica* were capable of pinocytosis; Feng, Feng, Burke and Khairallah (1971) also described the cytology, fine structure and cytochemistry of these cells. Martin (1970) studied the cytology and cytochemistry of hemocytes of *Spisula solidissima* and found only one type of cell, a granulocyte. Moore (1972), and Moore, Drake, and Eble (1972) described three types of hemocytes in *M. mercenaria,* a small agranulocyte and two types of granulocytes; they also reported on the cytochemistry of various enzymes, neutral lipids and glycogen. Gurski and Eble (1973) not only described hemocyte *in vitro* behavior but also reported cell counts for *M. mercenaria.* Loy and Eble (1974) described *in vitro* behavior as well as phagocytic aspects of *M. mercenaria* hemocytes as revealed by time-lapse cinematography. Foley and Cheng (1974) published on the cytology and aspects of the cytochemistry of hemocytes of *Mercenaria mercenaria;* they described three types of cells which they termed granulocytes, fibrocytes and hyalinocytes.

Investigation of defense mechanisms in invertebrates, particularly molluscs, was rekindled as a result of Stauber's (1950) pioneering work (Tripp, 1958a, b, 1961; Feng, 1959, 1966a, b; Bang, 1961; Cheng, Thakur and Rifkin, 1969). Hemocytes were found to phagocytose particulate foreign material and pinocytose soluble proteins (Feng, 1965); substances too large to be phagocytosed were surrounded by amoebocytes and encapsulated (Cheng, 1967; Feng, 1967; Cheng and Rifkin, 1970).

Reviews of molluscan hemocytes can be found in Wilbur and Yonge (1966), Cheney (1971) and Narain (1973).

Since there seems to be no general agreement on the various blood cell types in *M. mercenaria,* this paper attempts to delineate their cytology and aspects of their cytochemistry, as well as postulating a definitive classification of these cells. Further, a behavioral analysis of cell types are made based on time-lapse cinematography of cells.

MATERIALS AND METHODS

M. mercenaria was obtained from Buzzard's Bay, Massachusetts, and Great Bay, New Jersey. Clams were immediately used or maintained up to seven days in a 50 gallon aquarium kept at 22‰ salinity and 16° C.

Blood samples of approximately 0.01 ml were extracted from the ventricle by means of a 1 cc tuberculin syringe fitted with a number 30-gauge needle. Cells were allowed to settle on a cover glass for fifteen minutes in a moist chamber prior to observation or further preparations. Most cytological studies were done on living cells using the Zeiss Photomicroscope II, equipped with phase contrast and Nomarski interference phase optics.

Counts of cell types were made on a hemocytometer, using living cells over the course of one year. Cell types were identified on the basis of cell size and shape, number of cytoplasmic granules and proportion of hyaline cytoplasm, as well as size, position and characteristics of the nucleus. Percentage composition was determined by averaging differential counts of 100 cells using phase contrast optics. Measurements of cells and nuclei were done on living and fixed preparations using a calibrated ocular micrometer.

Hemocytes were supravitally stained by placing two to three drops of 1 : 20,000 concentration of Janus Green B, neutral red, or toluidine blue adjacent to the cells on a cover glass (Humason, 1972; Johnson, 1969); this technique enabled a gradual mixing of the stain and blood fluid which did not disturb the expanded cells. The cover glass was affixed to the slide by a rim of vaseline; this preparation was frequently viewed continuously for many hours without obvious deterioration of cells.

Hemocytes were fixed for two hours in buffered glutaraldehyde solution (3 ml of 50% stock glutaraldehyde, 47 ml of pH 7.5, 0.2 M phosphate buffer) and subsequently stained with Giemsa (1.0 ml of Giemsa stock, 2.0 ml of a 0.2 M phosphate buffer, pH 7.5, 47 ml of distilled water, Humason, 1972) to illustrate cellular morphology.

DNA was stained by the Feulgen reaction (Humason, 1972); hemocytes were fixed in Carnoy's fluid (3:1) or buffered glutaraldehyde. Vena's modification (1967) of the Feulgen reaction was also used.

TABLE I

Some morphological characteristics and percentage composition of hemocytes in Mercenaria mercenaria. *Mean sizes are for living cells. Percentage composition represents averaged differential counts of 100 cells. Approximately thirty animals were sampled* ($n = 300$ *of each cell type*).

Hemocyte type	Cell length (μm)	Nuclear diameter (μm)	Percentage composition
Agranulocyte	$\bar{x}$ = 5.0 s.d. = 0.68	3.2 0.55	2.0 2.1
Small granulocyte	$\bar{x}$ = 28.0 s.d. = 6.49	5.5 0.89	61.0 5.3
Large granulocyte	$\bar{x}$ = 45.0 s.d. = 7.30	8.5 1.12	37.0 6.1

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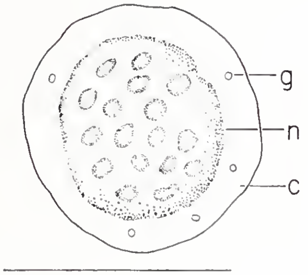

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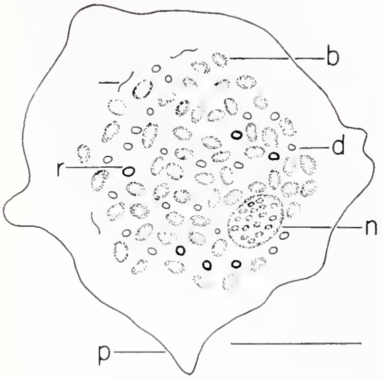

FIGURE 1. Diagram of a "typical" agranulocyte of *Mercenaria mercenaria.* Note the thin rim of cytoplasm surrounding the nucleus and few cytoplasmic granules: c, cytoplasm; n, nucleus; g, granule; 10 μm bar.

FIGURE 2. Diagram of a "typical" small granulocyte demonstrating four granule types confined to the endoplasm; b, blunt granule; d, dot-like granule; f, filamentous granule; n, nucleus; p, pseudopod; r, refractile granule; 9 μm bar.

The detection of both lipids and phospholipids employed hemocytes fixed two hours in buffered glutaraldehyde solution; cells were stained with Oil red 0 (Lillie, 1965) or Sudan black B (Humason, 1972), respectively.

Polysaccharides were revealed according to the periodic acid- Schiff (PAS) method of Lillie (1965). Several preparations were incubated 30 minutes in 0.5% alpha amylase solution before oxidation to identify glycogen; cells were pre-

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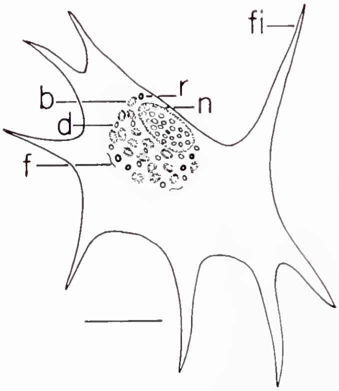
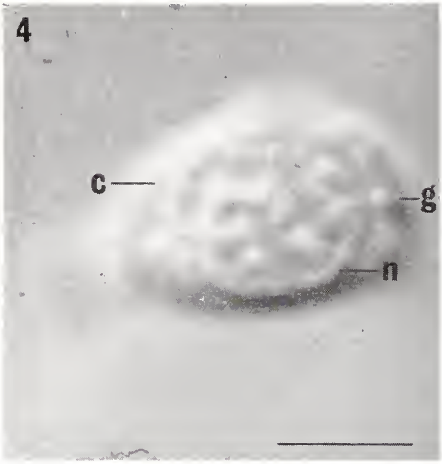


FIGURE 3. Diagram of a "typical" large granulocyte of *Mercenaria mercenaria.* Note the small number of granules as compared with the small granulocyte: b, blunt granule; d, dot-like granule; f, filamentous granule; fi, filopod; n, nucleus; r, refractile granule: 10 μm bar.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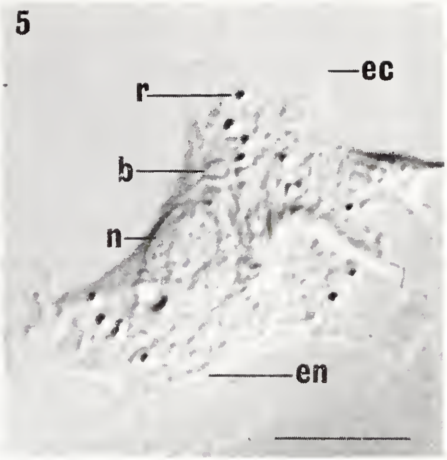
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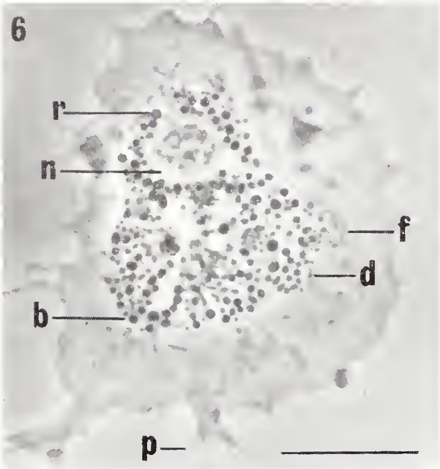
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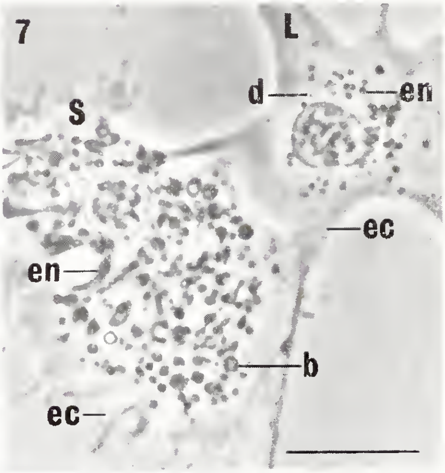
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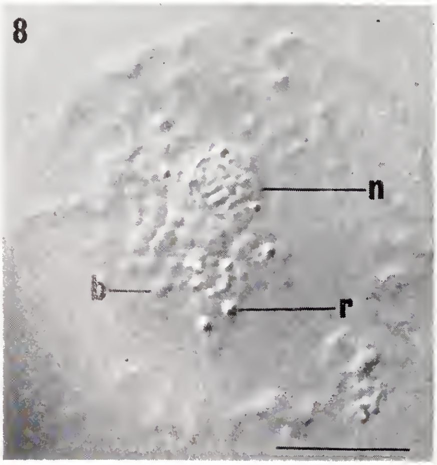
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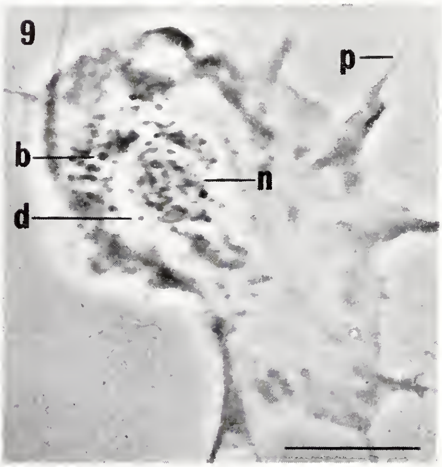
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viously fixed in Davidson's solution (two hours) or buffered glutaraldehyde solution (two hours). The distribution of polysulfated and polycarboxylated polysaccharides were studied in cells stained by the alcian blue pH 5.7 and alcian blue pH 2.6 methods of Mowry (Mowry, 1966; Scott, 1967). Blood cells fixed two hours in buffered glutaraldehyde solution were used in both preparations.

The Fairweather modification of Gomori's procedure was used to detect the presence of lipase activity (Chayen, Bitensky, Butcher and Poulter, 1969). All cells were previously fixed in cold (4° C) buffered glutaraldehyde solution (two hours). Detection of acid phosphatase was accomplished using two different techniques: in the first, cells were prepared according to Burstone's method (1958) after two hours fixation in 4% neutral formalin (4° C); and in the second, cells were fixed two hours in buffered glutaraldehyde solution (4° C) and processed following Goldberg's procedure (Goldberg, 1962; Goldberg and Barka, 1962). Controls were prepared by heat-inactivating cells as well as by omitting substrates from incubation solutions. Novikoff's method (1963) was used to demonstrate the presence of NADH dehydrogenase in cells fixed two hours in buffered glutaraldehyde solution (4° C). Nonspecific esterase was identified using Burstone's method (Pearse, 1962); cells were fixed for two hours in 10% neutral formalin.

Nuclear counterstaining was omitted since all preparations were examined with phase contrast optics which clearly delineated nuclear morphology.

Time-lapse studies were performed with a Zeiss Photomicroscope II fitted with a Sage time-lapse apparatus, Sage Series 500, Sage Instruments Inc., Model A, 16 mm Bolex camera with Plus-X reversal film was used to photograph specimens at an optimum speed of 30–40 frames per minute. Cells were prepared for viewing as for living preparations.

To demonstrate phagocytosis, yeast cells were washed in distilled water and affixed to a cover glass by heating. Hemocytes were placed next to yeast on the cover glass and placed in a moist chamber for 10–15 minutes. Preparations were then fixed in buffered glutaraldehyde solution and stained with Giemsa; some living preparations were also observed.

FIGURE 4. Agranulocyte as seen under Nomarski interference optics showing an extremely limited amount of cytoplasm and few granules. Note that nuclear morphology is similar to the other two cell types: c, cytoplasm; g, granule; n, nucleus; 3 μm bar.

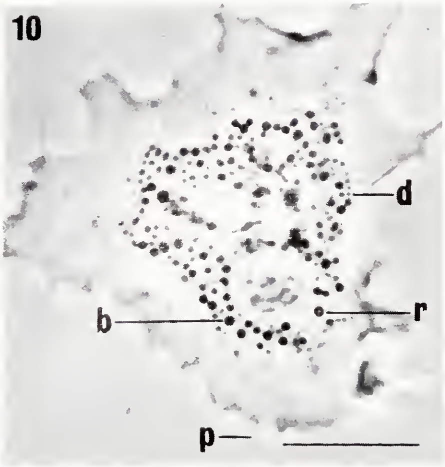
FIGURE 5. Small granulocyte of *Mercenaria mercenaria* viewed with Nomarski interference optics. Note numerous granules and limited ectoplasm: b, blunt granule; ec, ectoplasm; en, endoplasm; n, nucleus; r, refractile granule; 8 μm bar.

FIGURE 6. Small granulocyte supravitally stained with neutral red as seen under phase contrast optics: b, blunt granule, d, dot-like granule; f, filamentous granule; n, nucleus; p, pseudopod; r, refractile granule; 9 μm bar.

FIGURE 7. Large and small granulocytes stained with Janus Green B and viewed with phase contrast optics. Note number of granules present in both granulocytes: b, blunt granule; d, dot-like granule; ec, ectoplasm; en, endoplasm; L, large granulocyte; S, small granulocyte; 10 μm bar.

FIGURE 8. Living large granulocyte as seen under Nomarski interference optics: b, blunt granule; n, nucleus; r, refractile granule; 15 μm bar.

FIGURE 9. Large granulocyte supravitally stained with neutral red and viewed with phase contrast optics. Note uptake of dye by blunt and dot-like granules: b, blunt granule; d, dot-like granule; n, nucleus; p, pseudopod; 15 μm bar.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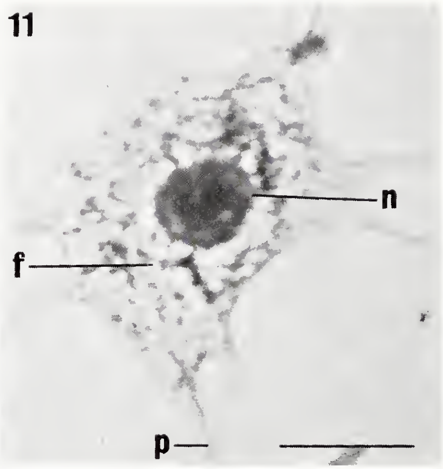
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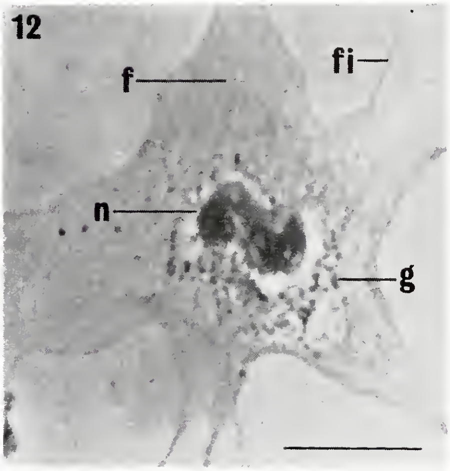
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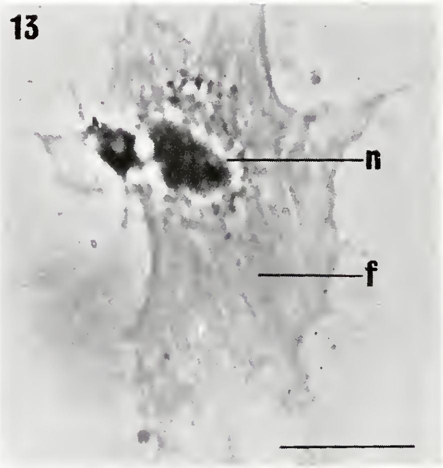
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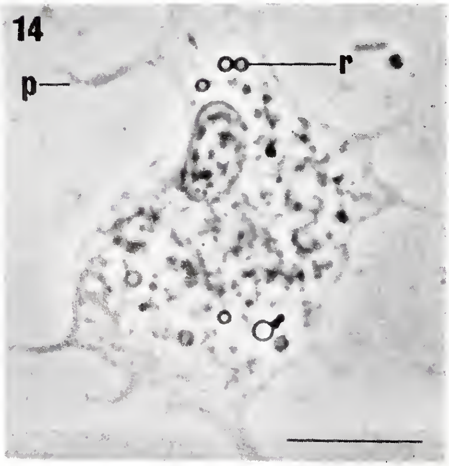
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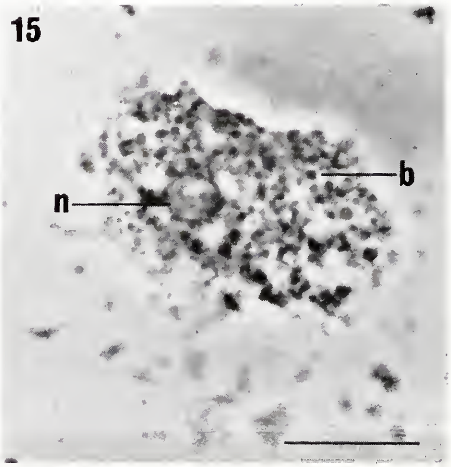
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TABLE II

Reactions of cytoplasmic granules to cytochemical techniques.

Technique	Blunt granule	Dot-like granule	Refractile granule	Filamentous granule
Giemsa	acidophilic	acidophilic	acidophilic	acidophilic
Janus Green B	+	+	—	—
Neutral red	red to amber	red to amber	some red to amber	—
Toluidine blue	γ-metachromasia	γ-metachromasia	—	—
NADH dehydrogenase	+	—	—	—
Acid phosphatase	—	+	—	—
Esterase	—	+	—	—
Sudan black B	outer boundary positive	entire granule positive	outer boundary positive	—
Oil red O	—	—	+	—
PAS	+	+	—	—
PAS after α amylase	+	+	—	—
Alcian blue 2.6	results were inconclusive			
Alcian blue 5.7	results were inconclusive			

RESULTS

Immediate examination of hemolymph shows blood cells to be rounded to oval. After fifteen minutes in a moist chamber most hemocytes adhere to glass and extend filopods. A detailed study of living and fixed hemocytes reveals three cell types: an agranulocyte and small and large granulocytes (Table I). These hemocyte types are present regardless of the organism's habitat.

Cell types

Agranulocyte. This cell averages 5 μm ± 0.68 in diameter with a prominent, centrally situated nucleus surrounded by a thin covering of cytoplasm practically devoid of granules. The rounded nucleus averages 3.2 μm ± 0.55 and has chromatin evenly dispersed throughout with a distinct rim of chromatin lining the nuclear membrane (Figs. 1 and 4). This cell shows no motility and comprises only 2% ± 2.1 of the total cell population.

Small granulocyte. The small granulocyte averages 28 μm ± 6.49 in its long

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FIGURE 10. Janus Green B-positive granules in a small granulocyte as seen under phase contrast optics: b, blunt granule; d, dot-like granule; r, refractile granule; p, pseudopod; 10 μm bar.

FIGURE 11. Small granulocyte stained with Giemsa. Note fibrous-like material in endoplasm: f, fibrous-like material; n, nucleus; p, pseudopod; 9 μm bar.

FIGURE 12. Giemsa-stained large granulocyte showing fibrous-like material in the ectoplasm: f, fibrous-like material; g, granule; fi, filopod; n, nucleus; 12 μm bar.

FIGURE 13. Giemsa-stained large granulocyte containing double nucleus. Note fibrous-like nature of ectoplasm; f, fibrous-like material; n, nucleus; 12 μm bar.

FIGURE 14. Lipid-filled refractile granules stained by Oil red 0 in a small granulocyte and viewed with phase contrast optics: p, pseudopod; r, refractile granule; 9 μm bar.

FIGURE 15. Localization of NADH dehydrogenase in blunt granules using phase contrast optics: b, blunt granule; n, nucleus; 12 μm bar.

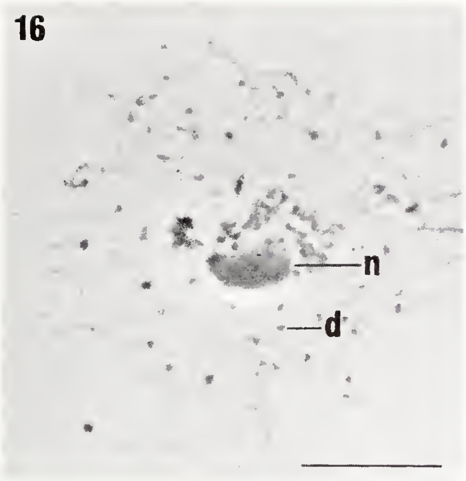
axis. The nucleus is oval, eccentric and averages 5.5 μm ± 0.89; nuclear cytology is similar to that of the agranulocytes (Figs. 2 and 5). The cytoplasm contains numerous granules in the endoplasm which can be classified into four types: a blunt, dot-like, filamentous, and refractile granule. Blunt granules, averaging 1.5 μm in length, are highly plastic (Figs. 6 and 7). These granules are most numerous, representing 52% of all granules. The dot-like granules, with an average diameter of 0.7 μm, comprise 33% of the granule population. Refractile granules (Figs. 5 and 6) are round with a prominent boundary. They average 1 μm in diameter and constitute 11% of the total number of granules. Filamentous granules are sparce, making up 4% of all granules and measure 2 μm in length. The small granulocytes possess a limited amount of clear ectoplasm in which some dot-like granules can be distinguished. This cell type is highly motile and moves in unidirectional patterns. The ectoplasm appears quite ruffled and forms filopods which adhere to the cover glass; occasionally an elongate filopodium forms and terminates in a web-like mass which adheres to the glass. Time-lapse studies show the margins of the small granulocyte to be in a constant state of wavelike motion. Adjacent granulocytes make contact by means of filopods forming a mesh of adhering cells. Granules within the cytoplasm are in constant motion and can be seen flowing from the cell body to the terminal mass. The small granulocyte comprises 61% ± 5.3 of the hemocyte population.

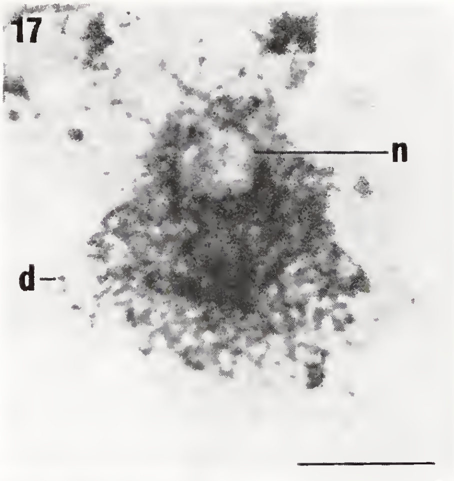
Large granulocyte. The large granulocyte averages 45 μm ± 7.30 in its long axis (Figs. 3 and 8). While nuclear position and morphology is similar to that of the small granulocyte, it is proportionally larger, averaging 8.5 μm ± 1.12 in length. The same four granule types are present in the cytoplasm, again confined to the endoplasm. Granules in large granulocytes are present in the same proportion as in small granulocytes but are only one-third as numerous (Fig. 7). The large granulocyte appears to possess limited mobility under light microscopy. Generally, this cell type spreads on the cover glass by means of filopods which considerably stretches the cytoplasm, giving the cell an extremely thin appearance (Figs. 7 and 9). Time-lapse photomicrography reveals a slow, erratic motion of this cell; the cell membrane and adjacent ectoplasm, however, are similar to the small granulocyte, in a continuous, waving motion. The granules in the endoplasm are also in a highly dynamic, erratic motion. Large granulocytes make up 37% ± 6.1 of the total cell population.

Supravital staining

Agranulocyte. No uptake of any vital stain used is observed in agranulocytes.

Small and large granulocytes. Blunt and dot-like granules (Fig. 10) of both granulocyte types take up and convert Janus Green B to the red-reduction product, diethyl safranin (Table II) within two hours. When exposed to neutral red, again dot-like and most blunt granules, with an occasional refractile granule, take up the dye in both small (Fig. 6) and large (Fig. 9) granulocytes. A color change from red to amber occurs from 30 seconds up to several hours, generally being slower in the larger granulocytes. Supravital studies with toluidine blue show similar metachromatic reactions in both granulocyte types (Table II), the large granulocytes again reacting slower.





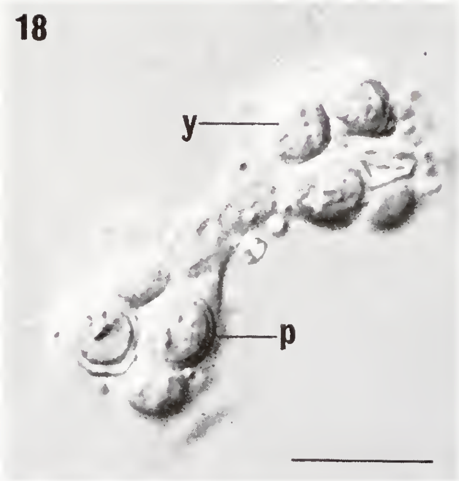


FIGURE 16. Acid phosphatase activity in dot-like granules as seen under phase contrast optics: d, dot-like granule; n, nucleus; 15 μm bar.

FIGURE 17. Nonspecific esterase activity in dot-like granules of small granulocyte viewed with phase contrast optics: d, dot-like granule; n, nucleus; 10 μm bar.

FIGURE 18. Yeast cells phagocytozed by a large granulocyte as seen with Nomarski interference optics: p, phagosome; y, yeast; 10 μm bar.

Cytochemistry

Giemsa. Observations of Giemsa preparations reveal the nucleus to stain basophilic, while cytoplasmic granules appear pink to red in both small (Fig. 11) and large (Figs 12 and 13) granulocytes. The agranulocyte was not observed.

Feulgen staining. All three hemocyte types show a similar nuclear morphology after staining with Feulgen. Dense areas of chromatin, uniformly distributed, stain deep violet. A dark staining rim of chromatin lines the nuclear membrane.

Lipids and phospholipids. Only refractile granules produce a lipid-positive reaction when stained with Oil red 0 (Fig. 14). Sudan black B is preferentially taken up by the outer boundary of the nucleus as well as the membranes of refractile and blunt granules in small and large granulocytes. Dot-like granules appear as solid black specks within the endoplasm and ectoplasm. Neither lipid nor phospholipid staining is seen in agranulocytes.

Polysaccharides. Periodic acid-Schiff reaction produces deep-red staining of dot-like and most blunt granules in small and large granulocytes. After treatment with δ-amylase, there is no reduction in staining intensity. Agranulocytes do not stain in this preparation. The detection of polysulfated and polycarboxylated polysaccharides proved inconclusive.

Enzymes. NADH dehydrogenase activity is localized in blunt granules (Fig. 15) of both small and large granulocytes. Agranulocytes appear to exhibit some activity, but this activity could not be identified in a distinct granule type. Acid phosphatase-positive granules are highly irregular with respect to shape and size (Fig. 16). Large and small granulocytes show 20–30 scattered granules, measuring slightly larger than dot-like but smaller than blunt granules. Nonspecific esterase staining of dot-like granules (Fig. 17) is observed in both granulocyte types. While the number of dye granules of acid phosphatase corresponds to the number of dot-like granules identified in living preparations, the number of dye granules of nonspecific esterase are much more numerous. Agranulocytes also demonstrate minute centers of nonspecific esterase activity in their limited cytoplasm.

DISCUSSION

Hemocytes of *Mercenaria mercenaria* may be divided into three types: agranulocytes, small granulocytes and large granulocytes. This classification is based on the following criteria: cell size, nuclear size, number of cytoplasmic granules, the ratio between endoplasm and ectoplasm, and motility.

The agranulocyte, with limited cytoplasm, has been identified by other investigators (Cuénot, 1891; Drew and Cantab, 1910; George and Ferguson, 1950; Dundee, 1953) in examining a variety of organisms. The original work on *Mercenaria mercenaria* hemocytes by Zacks (1955) describes only one cell type, a granulocyte. Later work by Foley and Cheng (1974) identified three classes of hemocytes but subsequently reduced this number to two (Cheng and Foley, 1975). The latter authors base their classification system on the presence of granules, as well as the possession of filopodia containing cytoplasmic fibers. Our studies have shown the large granulocyte capable of forming and absorbing filopodia containing cytoplasmic fibers in *in vitro* preparations. Hence, the fibrocyte and hyalinocyte types of Foley and Cheng (1974) appear to correspond to our large granulocyte. The cell we have classified as a small granulocyte is also referred to as a granulocyte by Foley and Cheng (1974).

Differential motility noted among hemocytes was also observed by Foley and Cheng (1974). The extreme motility of small granulocytes is due to a constant waving of ectoplasmic borders and advancing filopodia. Foley and Cheng (1974) describe a similar motility pattern for hemocytes designated as "granulocytes". Time-lapse studies (Loy and Eble, 1974) show that large granulocytes move by very slow, sliding motions with much waving of ectoplasmic borders; this appears to correspond to the motility of "agranulocytes" as explained by Foley and Cheng (1974).

The four granule types identified (blunt, dot-like, refractile and filamentous) exhibited specific reactions to the cytochemical techniques used (Table II). In Giemsa preparations the cytoplasm stains acidophilic, while the nucleus stains basophilic (Figs. 11 and 13). This was previously reported in *Mercenaria mercenaria* by Zacks (1955). The intense pink staining of cytoplasm and granules is contrary to findings by Foley and Cheng (1974), who report a network of blue inclusions and pale blue cytoplasm. Although individual granules are noted by Zacks (1955) and further delineated by Foley and Cheng (1974), no comprehensive study of granule types has been published.

Based on the ability of blunt granules to reduce Janus Green B to diethyl safranin, localization of NADH dehydrogenase activity in them (Fig. 15), their extreme plasticity (Lehninger, 1964), and boundary staining with Sudan black B, it can be concluded that these granules are mitochondria. Zacks (1955) designed "specific granules" as atypical mitochondria as a result of positive reactions to these cytochemical methods. Foley and Cheng (1974) found that nearly all cytoplasmic granules, with the exception of refractile inclusions, take up both Janus Green B and neutral red.

Localization of acid phosphatase produces very irregular but discrete centers of activity (Fig. 16). The fact that the granules measure slightly larger than the mean size of dot-like granules is attributed to possible accretion of dye due to the long incubation time (18 hours). Dot-like granules (Fig. 17) are also identified as centers of nonspecific esterase activity (Moore, Drake, and Eble, 1972). Zacks and Welsh (1953) found no esterase activity in *M. mercenaria* but indicated their histochemical experiments were not conclusive. Centers of nonspecific esterase activity are more numerous than those of acid phosphatase, indicating that many of these granules are very small or more permeable to reagents and not usually visible. The presence of nonspecific esterase in addition to acid phosphatase, which has been considered a marker for lysosomes (deDuve, 1963) suggests the lysosomal function of dot-like granules (Feng, Feng, Burke and Khairallah, 1971; Holt, 1963). Yoshino and Cheng (1976) have also identified centers of acid phosphatase activity in *Mercenaria mercenaria* as lysosomes. Supravital staining of dot-like granules by neutral red, thought to be indicative of lysosomes (Byrne, 1964a, b; Humason, 1972) also points to a lysosomal physiology. In addition, the uptake of Sudan black B demonstrates the presence of phospholipids, possibly as a membrane structure, typical of lysosomes (deDuve, 1963; Tappel, Sawant and Shibko, 1963).

Refractile granules demonstrate a positive reaction for lipids (Fig. 14), when stained with Oil red 0. The outer boundary staining of these granules with Sudan black B demonstrates the presence of phospholipids. On the basis of these reactions, it appears that refractile granules function as lipid storage centers.

The function of filamentous granules observed in living preparations is not clear since they are not seen to react with any of the cytochemical techniques. "Vermiform bodies" described by Foley and Cheng (1974) might correspond to this granule type.

Incorporation of bacteria into cytoplasmic vacuoles is occasionally observed in small and large granulocytes but never in agranulocytes. This suggests a phagocytic function for both granulocyte types of *M. mercenaria.* Work by Gurski and Eble (1973) shows small and large granulocytes phagocytizing yeast (Fig. 18) and three species of bacteria. Foley and Cheng (1975) have also found that hemocytes of *Mercenaria mercenaria* will phagocytize bacteria. Gurski and Eble (1973) demonstrated phagocytosis of yeast to be a direct function of yeast concentration. They further found small granulocytes to have a higher phagocytic rate than large granulocytes. Time-lapse cinematography (Loy and Eble, 1974) reveals phagocytosis in small granulocytes to occur by rapid cell extensions in and around the yeast, until the latter are incorporated within the cell as phagosomes. Large granulocytes however, phagocytose yeast cells by enveloping them with wave-like extensions of the outer ectoplasm.

It has been proposed by several authors (Cuénot, 1891; Drew and Cantab, 1910; Haughton, 1934; George and Ferguson, 1950) that various types of invertebrate hemocytes represent stages in the life cycle of one cell type. The high degree of nuclear morphological similarity in all three hemocytes of *M. mercenaria* supports this concept. Evidence is also offered by the presence of identical granule types in both small and large granulocytes. Furthermore, granules of granulocytes demonstrate similar reactions to all staining techniques. Based on these findings, it might be postulated that the agranulocyte represents the immature stage which gives rise to the small motile granulocyte upon formation of additional cytoplasm and granules; the large granulocyte may be the degenerate phase of the cycle with the cell gradually losing its motility as it undergoes degranulation. It is consistently observed that the granules of the large granulocyte take up supravital stains at a slower rate than corresponding granules of the small granulocyte. The fact that Loy and Eble (1974) found the large granulocyte to be a less active phagocyte than the small granulocyte also lends support to this hypothesis. Feng, Feng, Burke and Khairallah (1971) have described a senile stage in the hemocytes of *Crassostrea,* but nothing of this nature has been observed in *Mercenaria mercenaria.* Further work is required to determine whether the cell types described here are different development stages of a single cell line or if they are indeed separate and distinct cell lines.

SUMMARY

The hemocytes of the hard clam *M. mercenaria* were of three types: an agranulocyte, a small, and a large granulocyte. The agranulocyte, with only a thin periphery of cytoplasm surrounding the nucleus, had no visible cytoplasmic granules in living preparations but did exhibit a few centers of nonspecific esterase activity. This cell type represented 2% of the hemocyte population. The small granulocyte possessed four distinct granule types and comprised 61% of the total cell population. Large granulocytes accounted for 37% of all hemocytes. While they contained the same four granule types identified in the small granulocyte, only one-third the total number were present. The nucleus of all three hemocyte types appeared morphologically similar.

The four types of granules observed were a blunt, dot-like, a refractile and a filamentous granule. Blunt granules were identified as mitochondria, based on

their ability to reduce Janus Green B to diethyl safranin, the presence of NADH dehydrogenase activity and boundary staining with Sudan black B. Dot-like granules were identified as lysosomes on the basis of neutral red staining, localization of acid phosphatase and nonspecific esterase activity and staining with Sudan black B. Refractile granules were demonstrated to be membrane-bound, lipid-filled structures that reacted positively with Sudan black B and Oil red 0, respectively; these granules act as lipid storage centers.

Nuclear similarity of the three cell types suggest that these cells might represent different stages of maturity, rather than three distinct cell lines. This was also indicated by the similar yet graded cytochemical reactions and the varying degree of motility and phagocytic activity demonstrated by hemocyte types.

LITERATURE CITED

Bang, F. B., 1961. Reaction to injury in the oyster (*Crassostrea virginica*). *Biol. Bull.,* **121**: 57–68.

Byrne, J. M., 1964a. An electron microscopical study of neutral red granules in mouse exocrine pancreas. *Quart. J. Microsc. Sci.,* **105**: 219–225.

Byrne, J. M., 1964b. Acid phosphatase activity in neutral red granules of mouse exocrine pancreas. *Quart. J. Microsc. Sci.,* **105**: 343–348.

Burstone, M. S., 1958. Histochemical demonstration of acid phosphatase with napthol AS phosphates. *J. Nat. Cancer Inst.,* **21**: 523–539.

Chayen, J., L. Bitensky, R. G. Butcher, and L. W. Poulter, 1969. *A guide to practical histochemistry.* McGraw-Hill Book, Co., Inc., New York, 261 pp.

Cheney, D., 1971. A summary of invertebrate leucocyte morphology with emphasis on blood elements of the Manila clam, *Tapes semidecussata. Biol. Bull.,* **140**: 353–368.

Cheng, T. C., 1967. Marine molluscs as hosts for symbiosis: with a review of known parasites of commercially important species. Volume 5 in F. S. Russell, Ed., *Advances in marine biology.* Academic Press, New York.

Cheng, T. C., and E. Rifkin, 1970. Cellular reactions in marine molluscs in response to helminth parasitism. Pages 443–496 in S. F. Snieszko, Ed., *Symposium on disease of fishes and shellfishes.* American Fisheries Society Special Publication No. 5, Washington, D. C.

Cheng, T. C., A. S. Thakur, and E. Rifkin, 1969. Phagocytosis as an internal defense mechanism in the Mollusca: with an experimental study of the role of leucocytes in the removal of ink particles in *Littorina scabra* Linn. Pages 546–563 in *Proceedings of the symposium on Mollusca,* Part II. Marine Biological Association, India.

Cheng, T. C., and D. Foley, 1975. Hemolymph cells of the bivalve mollusc *Mercenaria mercenaria:* an electron microscopical study. *J. Invertebr. Pathol.,* **26**: 341–351.

Cuénot, L., 1891. Estudes sur le sang et les glands lymphatiques dans la serie animale. *Arch. Zool. Exp. Gen. Series 2,* **9**: 13–593.

deDuve, 1963. The lysosome concept. Pages 1–31 in A. V. de Reuck and M. Cameron, Eds., *Lysosomes, Ciba Foundation Symposium.* Little, Brown and Company, Boston, Massachusetts.

Drew, G. H., and B. A. Cantab, 1910. Some points in the physiology of lamellibranch blood-corpuscles. *J. Microsc. Sci.,* **54**: 605–621.

Dundee, D. S., 1953. Formed elements of blood of certain fresh-water muscles. *Trans. Amer. Microsc. Soc.,* **72**: 254–264.

Feng, S. Y., 1959. Defense mechanisms of the oyster. *Bull. N. J. Acad. Sci.,* **4**: 17.

Feng, S. Y., 1965. Pinocytosis of proteins by oyster leucocytes. *Biol. Bull.,* **129**: 95–105.

Feng, S. Y., 1966a. Experimental bacterial infections in the oyster *Crassostrea virginica. J. Invertebr. Pathol.,* **8**: 505–511.

Feng, S. Y., 1966b. Biological aspects of hard clam purification. *Proc. Nat. Shellfish Ass.,* **56**: 3.

Feng, S. Y., 1967. Responses of molluscs to foreign bodies with special reference to the oyster. *Fed. Proc.,* **26**: 1685–1692.

FENG, S. Y., J. S. FENG, C. N. BURKE, AND L. H. KHAIRALLAH, 1971. Light and electron microscopy of the leucocytes of *Crassostrea virginica* (Mollusca: Pelecypoda). *Z. Zellforsch. Mikrosk. Anat.*, **120**: 222–245.

FOLEY, D. A., AND T. C. CHENG, 1974. Morphology, hematologic parameters, and behavior of hemolymph cells of the quahaug clam, *Mercenaria mercenaria. Biol. Bull.*, **146**: 343–356.

FOLEY, D. A., AND T. C. CHENG, 1975. A quantitative study of phagocytosis by hemolymph cells of the pelecypods *Crassostrea virginica* and *Mercenaria mercenaria. J. Invertebr. Pathol.*, **25**: 189–197.

GEORGE, W. C., AND J. H. FERGUSON, 1950. The blood of gastropod molluscs. *J. Morphol.*, **86**: 315–324.

GOLDBERG, A. F., 1962. Acid phosphatase in leucocytes of normal patients with toxi-infectious states, infectious mononucleosis, eosiniphilias and auer bodies. *Fed. Proc.*, **21**: 74d.

GOLDBERG, A. F., AND T. BARKA, 1962. Acid phosphatase activity in human blood cells. *Nature,* **195**: 297.,

GURSKI, E., AND A. F. EBLE, 1973. Studies of blood cells of the hard clam, *Mercenaria mercenaria. Bull. N. J. Acad. Sci.,* **18**: 20.

HAUGHTON, I., 1934. XVII. Amœbocyte and allied cells in Invertebrata. *J. Roy Microsc. Soc.,* **54**: 246–262.

HOLT, S. J., 1963. Some observations on the occurrence and nature of esterase in lysosomes. Pages 114–120 in A. V. de Reuck and M. Cameron, Eds., *Lysosomes, Ciba Foundation Symposium.* Little, Brown and Company, Boston, Massachusetts.

HUMASON, G. L., 1972. *Animal tissue techniques.* W. H. Freeman and Company, San Francisco, 641 pp.

JOHNSON, P. T., 1969. The coelomic elements of sea urchins (*Strongylocentrus*). II. Cytochemistry of the coelomocytes. *Histochemie,* **17**: 213–231.

LEHNINGER, A. L., 1964. *The mitochondria.* W. A. Benjamin, Inc., New York, 263 pp.

LILLIE, R. D., 1965. *Histopathologic technic and practical histochemistry.* McGraw-Hill Book Co., Inc., New York, 715 pp.

LOY, N., AND A. F. EBLE, 1974. Locomotion and phagocytic behavior of the amoebocytes of the hard clam *Mercenaria mercenaria* as revealed by time-lapse cinematography. *Bull. N. J. Acad. Sci.,* **19**: 27.

MARTIN, A. S., 1970. Cytology, cytochemistry and enzyme cytochemistry of the amoebocytes of the Atlantic surf clam, *Spisula solidissima. Master's thesis, Trenton State College,* Trenton, New Jersey.

MOORE, C. A., 1972. Cytology and cytochemistry of the amoebocytes of the hard clam *Mercenaria mercenaria. Master's thesis, Montclair State College,* Montclair, New Jersey.

MOORE, C. A., N. DRAKE, AND A. F. EBLE, 1972. Cytology and cytochemistry of the amoebocytes of *Mercenaria mercenaria. Bull. N. J. Acad. Sci.,* **17**: 44.

MOWRY, R. W., 1966. The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study for mucins. With revised directions for the collodial iron stain, the use of alcian blue G8X and their combinations with the periodic acid-Schiff reaction. *Ann. N. Y. Acad. Sci.,* **106**: 402–423.

NARAIN, A. S., 1973. The amoebocytes of lamellibranch molluscs, special reference to the circulating amoebocytes. *Malacol. Review,* **6**: 1–12.

NOVIKOFF, A. B., 1963. Electron transport enzymes: biochemical and tetrazolium staining studies. *First International Congress of histochemistry and cytochemistry* (R. Wegmann, Ed.). Pergamon Press, New York, 481 pp.

PEARSE, A. G., 1962. *Histochemistry-theoretical and applied.* Little, Brown and Company, Boston, Massachusetts, 998 pp.

SCOTT, J. E. (Scott's method as used by Mowry), 1967. Observations in basophilia of amyloids. *Histochemie,* **10**: 8–32.

STAUBER, L. A., 1950. The fate of india ink injected intracardially into the oyster, *Ostrea virginica* Gemelin. *Biol. Bull.*, **98**: 227–241.

TAKATSUKI, S., 1934. On the nature and function of amoebocytes of *Ostrea edulis. Quart. J. Microsc. Sci.,* **76**: 379–431.

TAPPEL, A. L., P. L. SAWANT, AND S. SHIBKO, 1963. Lysosomes: distribution in animals, hydrolytic capacity and other properties. Pages 78–108 in A. V. de Reuck and

M. Cameron, Eds., *Lysosomes, Ciba Foundation Symposium.* Little, Brown and Company, Boston, Massachusetts.

TRIPP, M. R., 1958a. Disposal by the oyster of intracardially injected red blood cells of vertebrates. *Proc. Nat. Shellfish. Ass.*, **48**: 143–147.

TRIPP, M. R., 1958b. Studies on the defense mechanism of the oyster. *J. Parasitol.*, **44** (sect. 2): 35–36.

TRIPP, M. R., 1961. The fate of foreign materials experimentally introduced into the snail *Australorbis glabratus. J. Parasitol.*, **47**: 745–751.

VENA, J., 1967. Feulgen reaction at room temperature. *Turtox News*, **45**: 252.

WILBUR, K. M., AND C. M. YONGE, 1966. *Physiology of Mollusca, Volume II.* Academic Press, New York, 645 pp.

YOSHINO, T., AND T. C. CHENG, 1976. Fine structural localization of acid phosphatase in granulocytes of the pelecypod *Mercenaria mercenaria. Trans. Amer. Microsc. Soc.*, **95**: 215–220.

ZACKS, S. I., 1955. The cytochemistry of the amoebocytes and intestinal epithelium of *Venus mercenaria* (lamellibranchiate), with remarks on a pigment resembling ceriod. *Quart. J. Microsc. Sci.*, **96**: 57–71.

ZACKS, S. I., AND J. H. WELSH, 1953. Cholinesterase and lipase in amoebocytes, intestinal epithelium and heart muscle of the quahog, *Venus mercenaria. Biol. Bull.*, **105**: 200–211.

Reference: *Biol. Bull.*, **152**: 120–133. (February, 1977)

AVAILABILITY AND USE OF SHELLS BY INTERTIDAL HERMIT CRABS

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A typical hermit crab protects its soft parts by enclosing them in a gastropod shell. Empty shells are often durable enough that one could provide protection for a crab's entire lifetime. However, crabs choose shells that fit their bodies closely, and crabs cannot continue to grow unless they have a continuous supply of shells [except *Pagurus prideauxi* (Pike and Williamson, 1959) *P. bernhardus* (Jensen, 1975), and numerous other species (Nyblade, 1974) whose shells support colonial organisms that grow continuously forward from the lip of the shell].

If hermit crabs require a continuous supply of shells, then the shell supply rate may influence crab numbers. An opportunity to test this hypothesis arose during long-term studies of snail populations on a rocky shore area, Shady Cove, 0.5 mile north of Friday Harbor Laboratories, San Juan Island, Washington. Between August 1967 and August 1970, 4272 specimens of *Thais lamellosa* and 582 of *T. emarginata* from the 200 m^2 study area were given individually numbered tags (Spight 1974). About 40% of the snails died each year, and as snails died, their tagged shells were acquired by the hermit crabs of the vicinity. The first crab with a tagged shell was found during May, 1968, and progressively more were found in succeeding months. Since the crabs used many of the tagged shells, the crabs provided a valuable means to verify snail deaths, and deliberate censuses of tagged crabs were undertaken. These census data will be examined here for correlations between changes in the rate of shell supply and changes in the portion of the crab population using shells of *Thais*.

METHODS

Censuses

Censuses were taken from August, 1967, until May, 1973. Many of the early censuses covered irregular portions of the study area. After 1968 the entire 200 m^2 area of Shady Cove (see map in Spight, 1974) was searched regularly. Two types of searches were conducted: either each portion of the study area was visited once and the number of each shell or snail encountered was noted (note: the word *shell* indicates a shell occupied by a crab; shells occupied by snails are indicated by the word *snail*). Otherwise, each portion of the study area was visited on two consecutive days, and, on each day, all snails and shells encountered were removed. When a shell was removed from the field, it was measured, and the species of crab occupying it was determined. If the shell was intact, it was then returned. If the shell was broken, an intact shell was provided, and the crab was returned to Shady Cove with the new shell.

Population size

One-day censuses usually did not include all of the hermit crabs in the population because only tagged shells were noted. No collections were undertaken specifically to determine the number of untagged shells being used by the crabs. However, shells without tags were counted during some censuses and these counts can be used to estimate how many untagged shells were typically used. The tagged fraction (P) will be expressed as the ratio of the number of tagged shells to the total number of shells collected.

Tagged shells were introduced during 1969. This introduction affected the tagged fraction and, therefore, uncorrected population estimates. The study area was visited six times between May 1 and 14, and on each visit all crabs were removed from a large covered tidepool (K-9). In the laboratory, all crabs wearing tagless shells traded their shells for tagged ones. The crabs with shells of *T. lamellosa* were returned to Shady Cove on May 18, and those with shells of *T. emarginata* were returned on June 18, 1969.

The tagged fractions also reflect the extent of the snail tagging program. All *T. lamellosa* found on the study area from May, 1969 to August, 1970, were either tagged or removed. In contrast, tags were regularly issued to *T. emarginata* from only about a quarter of the study area (C_u of Spight, 1974).

The census data also underestimate the number of hermit crabs because some tagged shells are not seen. The study area was thoroughly searched during consecutive days on 11 different occasions. The total number of tagged shells (N_m) can be estimated with the data obtained. If the same proportion, k, of the shells was found on the beach each day [*e.g.*, (collection of day 1) $= N_1 = kN_m$ and (collection of day 2) $= N_2 = k(N_m - N_1)$], then k can be found from: $k = 1 - N_2/N_1$.

Hermit crab numbers were obtained from 1-day census totals. It was assumed that k of the crabs without tags were found as well as k of those with tags. (Tags are not as obvious as shells, and all shells found were collected to maximize the number of tags found.) Therefore, the total crab population (N_c) for any date can be obtained from $N_c = N_1/kP$. For shells of *Thais emarginata*, overall averages ($k = 0.622$, $P = 0.609$), were used for all dates. For shells of *T. lamellosa,* the same first-day fraction was used for all dates ($k = 0.733$), but different tagged-fractions were used to reflect the effects of shell additions (a separate P calculated for each date, May-October, 1969, and the average figure, $P = 0.717$, for all dates after October, 1969). The separate population estimates for the two shell species were then summed to obtain the total number of hermit crabs.

Shell availability

The number of shells presumed to be available to the crabs is the number of snails seen alive for the last time during the previous census month. The sources of these numbers are given in detail in Spight (1972); actual numbers have been updated here to reflect censuses taken to and including May 5, 1973. Shell availability was calculated as follows (using June, 1969, as an example): 131 individuals of *Thais lamellosa* were seen for the last time during May 1969. Shells of these snails were assumed to have become available to the crabs before June 26

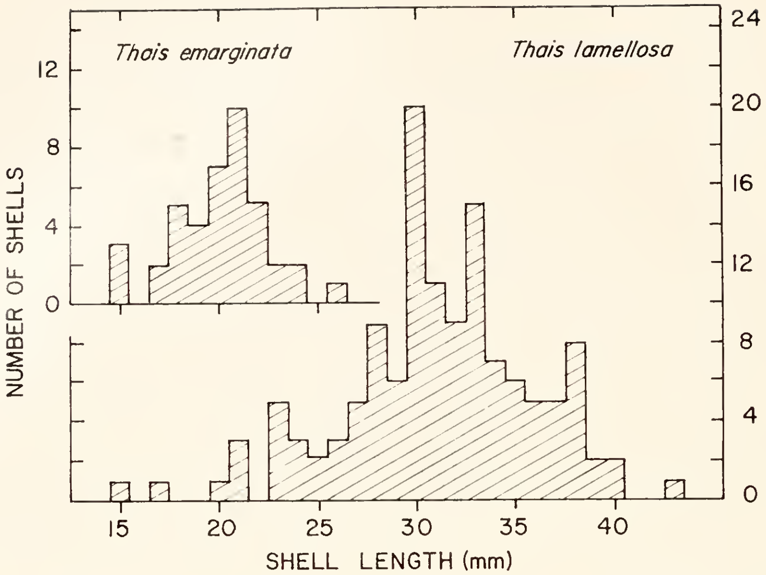

FIGURE 1. Sizes of shells of two snail species. Shells were worn by hermit crabs and were collected on the shore at Shady Cove on September 20, 1969.

(the first snail census date in June) at a rate of 131 shells per 27 days, or 4.8 shells per day. These were available to the 130 crabs present on May 30. Therefore (4.8 × 30)/130 = 1.12 shells per crab-month were available. Based

TABLE I

Sizes of shells used by Pagurus granosimanus *at Shady Cove, San Juan Island, Washington, on various dates. Species determinations were not made on August 5, 1972; all data are included.*

Date	Shell species						Mean total shell length		
	Thais lamellosa Mean shell length			*Thais emarginata* Mean shell length					
	N	mm	s.d.	N	mm	s.d.	N	mm	s.d.
1969									
September 20	129	31.0	4.87	41	20.1	2.41	170	28.4	6.43
November 8	108	30.8	4.94	31	20.9	2.11	139	28.6	6.07
1970									
April 9	42	29.6	5.57	12	20.8	1.64	54	28.7	6.19
July 14	38	29.9	5.99	22	21.2	2.26	88	27.7	6.52
October 15	33	30.4	4.97	12	21.2	2.52	45	27.9	6.05
1971									
April 26	45	28.4	6.19	23	21.1	2.59	68	25.9	6.29
1972									
April 13	8	27.6	2.39	1	22	—	9	27.0	2.92
August 5	10	30.9	7.40	3	21.7	0.58	13	28.5	8.48

on analogous data and calculations, 0.26 shells of *T. emarginata* also became available for a total of 1.38 shells per crab-month.

These numbers exaggerate shell availability. Some shells are crushed when crabs (*Cancer* spp.) kill snails, and others are removed from the study site by birds (Spight, 1976). Other snails may be below the intertidal zone when then they die, and their shells will only become available to intertidal crabs through trades with subtidal crabs. However, as long as snails die in the same ways year after year, snail death rates should be a good index of shell availability. When crabs were counted most frequently, and were most abundant, 60% of available shells were used. Therefore, most snail shells are accessible to intertidal crabs when the snails die.

Results

Shells used

Hermit crabs use the shells of 12 snail species at Shady Cove. Number tags were put on shells of three species of intertidal snails (*Thais lamellosa, T. emargi-*

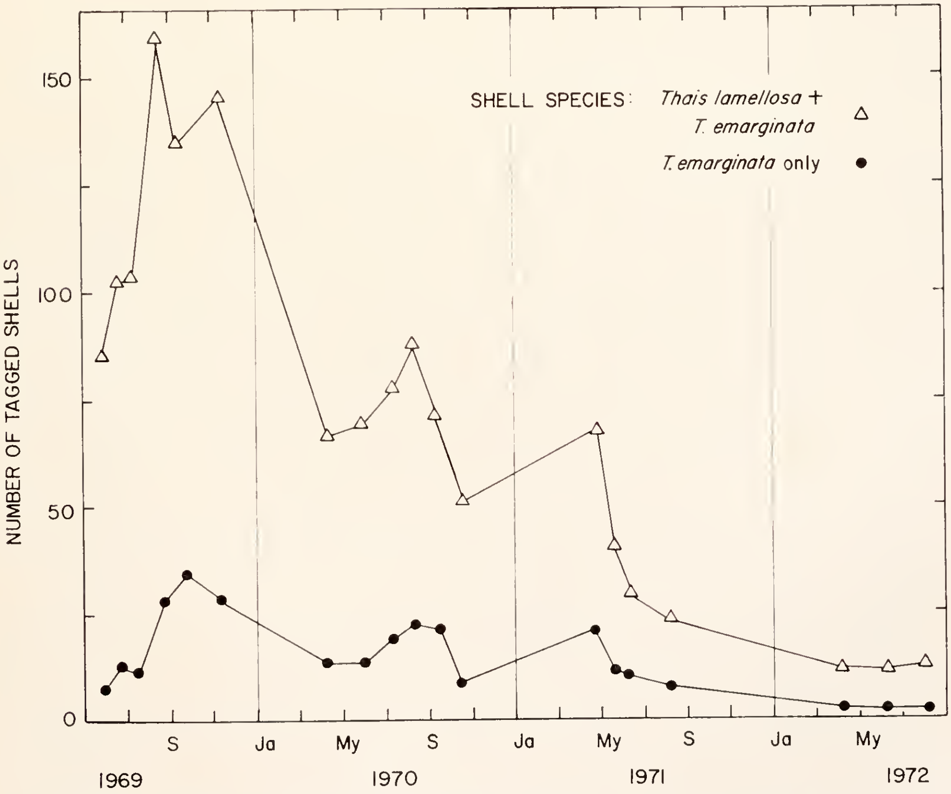

FIGURE 2. Number of hermit crabs found in tagged shells of *Thais lamellosa* and *T. emarginata,* on various dates, 1969–1972 (first-day only).

TABLE II

Species of hermit crab inhabiting shells of Thais lamellosa *and* T. emarginata *at Shady Cove, San Juan Island, Washington: B,* Pagarus beringanus; *G,* P. granosimanus; *and H,* P. hirsutiusculus. *The table includes crabs without tags if these were collected.*

Date of census	Number of crabs inhabiting shells of					
	Thais emarginata			*Thais lamellosa*		
	G	B	H	G	B	H
1969						
September 20-21	41	0	7	132	2	2
October 20	21	0	0	80	0	0
November 8	30	0	8	97	21	4
November 9–11	1	0	0	10	68	0
1970						
April 9–12	23	0	7	64	1	3
July 14	25	0	2	70	5	2
October 15	15	0	5	51	18	2
1971						
April 26	24	0	0	45	2	1
1972						
April 13	1	0	2	8	0	0
Total	181	0	31	557	117	14

nata, and *Searlesia dira*). The crabs used *T. lamellosa* shells 12–47 mm shell length, and *T. emarginata* shells 15–23 mm shell length (Fig. 1). These two shell species provide a vast majority of the shells 15 mm and larger. Tagged *S. dira* shells comprised only 1–6% of the population of tagged shells, and crabs used only a few of the tagged shells that became available; perhaps the crabs avoid the thin *S. dira* shells. Medium to large crabs also use shells of *Margarites pupillus, Calliostoma ligatum, Amphissa columbiana, Bittium eschrichtii, Ocenebra lurida, O. interfossa,* and *T. canaliculata.* Of these species, only *M. pupillus* is abundant at the site, and together these seven shell species provide only a small fraction of the shells (10 mm shell length or more) used by medium to large crabs. The smallest crabs use shells of *Littorina* and *Lacuna* (6–10 mm shell length).

During the present study, only crabs in *Thais* shells were counted, and thus data only provide information on larger hermit crabs. In this paper, the "shell population" includes only *T. emarginata* and *T. lamellosa* shells, even though a few large and all small crabs are excluded.

Most of the crabs in the population were fairly large. *T. emarginata* provided most shells smaller than 25 mm (Fig. 1), but these usually formed only 20–30% of the shell population (Fig. 2). To identify any trends in crab size, mean shell length was calculated for all censuses on which shells had been removed from the field (Table I). The largest and smallest means differ by only 2.8 mm (±5%), and no seasonal or longer term trends are evident. Crabs wearing shells 10–18 mm were never a conspicuous component of the crab population (*e.g.,* Fig. 1).

Species of hermit crabs

Three hermit crab species have been collected at Shady Cove: *Pagurus granosimanus, P. hirsutiusculus,* and *P. beringanus. P. granosimanus* and *P. hirsutiusculus* are generally found under stones during low tide periods. *P. beringanus* does not normally remain in the exposed portions of the tidal zone during low water periods (see also Vance, 1972a). Individuals were occasionally obtained from a covered tide pool (K-9) but were otherwise obtained only by searching at the water line during night low tides (October to February; with the mixed tidal cycle of the San Juan Islands area, the lower shore is generally exposed only once per day).

The relative abundances of these crab species (in *Thais* shells) were obtained by identifying the occupants of all shells removed from the field. Of the 900 crabs

Table III

Percentage of the hermit crabs from Shady Cove that were wearing tagged shells. An entry is included for all dates on which shells without tags were noted on the data books. The collection total for a province is included only if untagged shells were noted.

Date of census	*Thais lamellosa* shells		*Thais emarginata* shells	
	Number seen	Per cent with tags	Number seen	Per cent with tags
1969				
May 1	63	61.9	2	50.0
May 1–14	105	55.2	17	41.1
June 18	77	96.1		
July 1	10	70.0		
July 11–17	96	93.7		
July 29	77	88.3		
August 26	67	85.0	25	88.0
September 20–21	177	77.4	72	66.7
October 20	88	84.0	29	65.5
November 8–9	196	69.8	73	46.5
December 8	12	75.0		
1970				
April 9	95	65.2	34	61.7
May 23	40	70.0	10	50.0
May 31	43	76.7		
June 16	34	79.4	3	33.3
July 14	76	75.0	25	56.0
October 15	70	75.7	17	76.4
1971				
April 3	24	70.8	2	50.0
April 26	38	71.0	30	60.0
May 12	33	75.7	10	80.0
May 23	24	75.0	11	63.6
June 7	15	66.7		
1972				
April 13	5	60.0		
1973				
May 4	6	33.3	20	5.0
Total	1402	76.3	35.8	60.9

identified, 82% were *Pagurus granosimanus,* 13% were *P. beringanus,* and 5% were *P. hirsutiusculus* (Table II). The crabs used the two shell species in different proportions. *P. hirsutiusculus* was found disproportionately more frequently in shells of *T. emarginata* (*e.g.,* the smaller shells; Fig. 1), and *P. beringanus* was found only in shells of *T. lamellosa* (*e.g.,* the larger shells; Table II). This usage pattern may reflect shell size rather than shell-species; it parallels the observations of Vance (1972a). The conclusions of this paper are thus based primarily on data for *P. granosimanus.*

Population changes

Many more tagged shells were found on the study area at some times than at others. The crab population doubled between May and August, 1969, and numbers remained high through the fall of 1969. Numbers declined abruptly during the winter, and the decline progressed more or less continuously until the end of the observation period (Fig. 2). In the final census of May, 1973, only 26 crabs were observed, of which only 3 wore tagged shells.

The crabs used untagged shells as well as tagged ones. Overall, about 76% of the shells of *Thais lamellosa* and about 61% of the shells of *T. emarginata* encountered on censuses were tagged (Table III). For *T. lamellosa* shells, the tagged fraction rose from 62% to 96% after the addition of shells in May, 1969, and then gradually decreased to around 70% (mean, for all dates, November 1969 to April, 1972, 71.7%). In June, 1969, 19 shells of *T. emarginata* were added

TABLE IV

Number of hermit crabs in tagged shells found on consecutive searches at Shady Cove.

Date	Total number of crabs found			
	Crabs in *Thais lamellosa* shells		Crabs in *Thais emarginata* shells	
	First day	Second day	First day	Second day
1969				
April 16	18	1	3	0
July 11	92	20	12	2
September 20	100	39	35	16
November 9	118	37	28	9
1970				
April 9	54	10	13	10
July 14	59	22	19	8
October 15	42	10	9	5
1971				
April 26	48	7	20	4
1972				
April 13	10	0	2	0
August 5	11	2	2	0
1973				
May 4	2	0	0	0
Total	554	148	143	54

TABLE V

Estimated crab population and number of shells available. Census totals are numbers of crabs seen on first-day censuses, given by shell species (L, Thais lamellosa; *E,* T. emarginata*). Estimated crab population includes untagged shells of these two snail species (see text). Number of shells available is based on the number of snails seen for the last time on the date listed, the number of crabs seen on that date, and the number of days to the next date; it is given for each shell species.*

Date	Number of crabs seen on census		Estimated crab population	Number of shells available per crab per month		
	L	E		L	E	Total
1969						
May 30	78	7	130	1.12	0.26	1.38
June 26	90	13	162	0.67	0.12	0.79
July 11	92	12	166	0.73	0.26	0.99
August 25	132	28	286	0.49	0.14	0.63
September 20	100	35	268	0.63	0.07	0.70
November 8	118	28	298	0.15	0.01	0.16
1970						
April 11	54	13	136	0.78	0.11	0.89
May 31	55	14	142	0.68	0.25	0.93
July 14	59	19	162	0.69	0.23	0.92
August 12	65	23	185	0.65	0.12	0.77
September 10	50	21	151	0.36	0.16	0.52
October 15	42	9	104	0.08	0.02	0.10
1971						
April 26	48	20	144	0.88	0.08	0.96
May 23	29	11	84	0.45	0.02	0.47
June 7	20	10	64	0.41	0.03	0.44
August 5	16	7	49	0.01	0.00	0.01
1972						
April 13	10	2	23	1.36	0.02	1.38
June 9	10	2	23	0.95	0.00	0.95
August 5	11	2	26			

to the 13 known to have been in the population, and the tagged fraction subsequently rose from 41% to 88%. The tagged fraction dropped abruptly to 67% in September, and remained at about that level until May, 1971 (Table III). The lower tagged fraction for *T. emarginata* shells reflects the lower proportion of tagged snails on the study area (see Methods).

Not all of the tagged shells were seen on every census. Overall, 21% of the shells of *T. lamellosa* and 27% of the shells of *T. emarginata* were found on the second day's search (Table IV). These data indicate that 73% of the tagged *T. lamellosa* shells at large and 62% of the tagged *T. emarginata* shells at large were seen on the average census ($k = 0.733$ and 0.622, respectively; see Methods).

The untagged shells and tagged shells that were missed together amount to almost as many as were included in the first-day counts of tagged shells (Table V).

Availability and use of shells

During most months, 0.5 to 1 new shells became available for each crab in the population (Table V). Overall, 24% of the *T. emarginata* shells and 40%

Table VI

Availability (snail deaths, or number of snails seen alive for the last time during the month indicated) and use of shells of two snail species by hermit crabs of Shady Cove, San Juan Island, Washington. All snails dying before or not encountered during the final census, May, 1973, are listed as deaths in August, 1972.

Month	*Thais lamellosa*			*Thais emarginata*		
	Snail deaths	Shells used	Per cent	Snail deaths	Shells used	Per cent
1967						
August	1	0	0	0	—	—
September	20	4	20	13	1	8
October	8	2	25	16	1	6
December	16	3	19	4	3	75
1968						
January	5	1	20	0	—	—
February	14	7	50	0	—	—
April	82	27	33	5	2	40
May	42	18	43	10	4	40
June	95	46	48	8	2	25
July	73	42	57	10	2	20
August	142	48	34	63	25	40
September	104	35	35	8	5	62
October	118	56	47	16	2	12
November	15	7	47	19	1	5
1969						
January	4	4	100	0	—	—
April	116	25	22	11	3	27
May	131	61	46	31	15	48
June	54	19	35	10	7	77
July	183	70	38	65	35	54
August	121	33	27	35	24	69
September	278	35	13	29	15	52
November	233	11	5	14	3	21
1970						
April	176	30	17	25	6	24
May	54	20	37	17	4	23
June	87	24	28	35	13	37
July	108	17	16	36	21	58
August	117	13	11	22	8	36
September	63	7	11	29	4	14
October	55	7	13	12	3	25
1971						
April	114	7	6	11	4	36
May	19	0	0	1	0	0
June	52	2	4	4	1	25
August	5	1	20	0	—	—
1972						
April	60	1	2	1	1	100
June	42	0	0	0	—	—
August	169	1	1	0	—	—
Total	2976	684	23	560	215	38

TABLE VII

Observed use of Thais *shells by hermit crabs at Shady Cove as a function of the number of censuses in the months following the death of the snail.*

Shell species	*Thais lamellosa*				*Thais emarginata*			
Number of censuses taken within two months of snail disappearance	0	1	2	3	0	1	2	3
Number of months of observations	5	7	10	7	5	7	10	7
Number of shells available	293	327	1100	895	26	70	238	188
Percentage of shells used	6.5	22.3	27.1	27.4	23.1	12.9	41.5	50.0

of the *T. lamellosa* shells that became available from May 1969 through April 1972 are known to have been used by the crabs (Table VI). More of the available shells were used during 1969 than during later years.

The use frequencies reflect both census frequency and crab population size. More uses were detected when several censuses were conducted shortly after the death date (*e.g.*, 1969; Table VII). Available shells were more often used when many crabs were on the study area (Fig. 3).

DISCUSSION

Hermit crab populations are believed to be limited by shell supplies by many marine biologists. Four kinds of data have been presented to support this hypothesis. First, all accessible shells are occupied; no empty shells are available

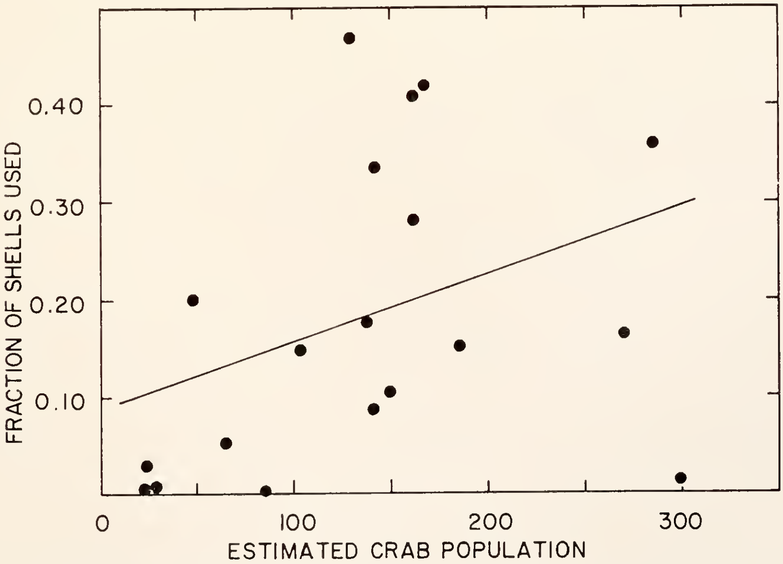

FIGURE 3. Shell use by hermit crabs: use of available shells at different crab densities.

to provide protection for additional crabs (Provenzano, 1960; Childress, 1972). Secondly, many crabs have small or broken shells which they will readily trade for larger or unbroken shells (Vance 1972b). Thirdly, larger crabs become abundant when larger shells become available (Drapkin, 1963). Fourthly, crab populations have increased when shell supplies have increased (Vance, 1972a). Conspicuously absent from this list are any references to the normal components of arguments about population limitation: measurements of the availability of resources (the rate of shell supply) or of crab population parameters (growth or reproductive rates). Furthermore, each of the existing kinds of data provides, at best, ambiguous support for the proposition.

In most environments, the number of shells will be equal to the number of hermit crabs because unoccupied shells are removed by physical processes. Empty shells will roll down the shore until they reach pockets of soft or loose sediment. The shells will then be buried by the shifting sediment and will no longer be available to crabs. Hermit crabs compete with this physical removal and are virtually the only means for keeping a shell on the surface. Therefore, available shells cannot be more numerous than hermit crabs in most communities.

Most crabs would avoid using a small or a broken shell if an alternative were available and have good reasons for doing so. Crabs in inadequate shells are more susceptible to predators (Vance 1972b), produce fewer eggs (Childress, 1972), and grow more slowly (Markham, 1968; Nyblade, 1974). If crabs use poor shells, then good shells must be in short supply, and survival, growth and reproduction may be limited by the number of high-quality shells available. Drapkin (1963) provides a clear example of this type of shell limitation. When a larger shell species became available in the Black Sea (*Rapana bezoar*), its shells were readily accepted by the small hermit crabs then present, and hermit crabs began attaining sizes that had not been seen prior to the introduction. The best estimates of shell adequacy for whole populations are those of Vance (1972a); most large crabs from his population preferred shells larger than their own. None of these examples show that crab numbers are limited by shell supply. In Vance's study, unoccupied small shells were found in nearby tidepools; if these shells were available to crabs, then recruitment and density were not limited by the shell supply. Most larger crabs had inferior shells, and presumably were producing fewer gametes than if better shells had been available. However, Nyblade's (1974) settlement experiments indicate that small shells would be used as long as they are accessible to the larvae.

Vance (1972a) attempted to increase crab numbers on a small isolated reef by adding 12,000 shells. This experiment provides some of the most compelling evidence for shell limitation available to date. However, the rate of increase was low (0.094 crabs per shell added), the experiment was unreplicated (the scale of the effort precluded replication), and year-to-year changes in unmanipulated natural populations have not been typified. Since typical rocky shore populations vary greatly (Spight 1974), Vance's result could well have arisen by chance.

The present data allow a different approach to the shell-limitation problem. A hermit crab (*Pagurus granosimanus*) population was monitored over a relatively long time (Fig. 2). Over the same period, death rates of the snails that furnished the shells were monitored. Changes in the crab population can be compared with changes in shell availability.

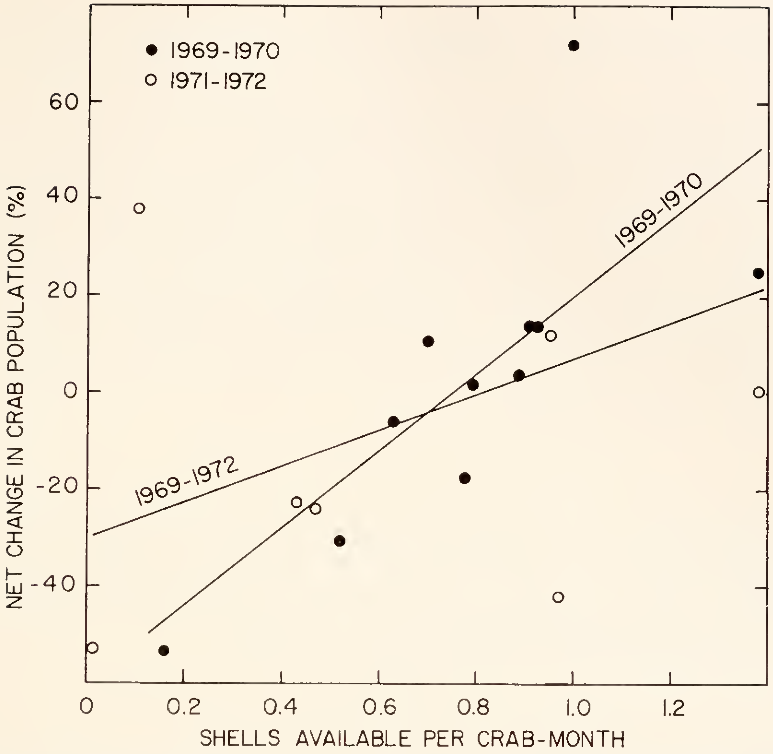

FIGURE 4. Observed changes in the crab population related to the number of *Thais* shells that became available during the period.

The shell supply changed seasonally. Many new shells became available during the spring and summer, and few were available during the winter (Table VI). These seasonal pulses in shell availability were reflected in 1969 and 1970; the crab population increased during summer and fall and decreased during winter.

Shell supplies also changed over the longer term. The snail populations at Shady Cove declined almost continuously during the period of study (Spight, 1972). Correspondingly, crab numbers also declined steadily after reaching a peak in 1969 (Fig. 2). These parallel courses in crab and snail populations are consistent with the hypothesis that crab numbers are affected by the shell supply.

Crab population size was related more directly to shell availability by plotting the net change in the crab population ($N_{t+1} - N_t$, from Table V) against the rate of shell availability (number of new shells per crab-month, from the crab population of Table V, and the number of snails seen for the last time, Table VI). When numbers were changing greatly (1969–1970), variations in shell supply accounted for 59% of the variations in crab numbers ($r^2 = 0.59$; Fig. 4). The correspondence is less for the whole period (1969–1972; $r^2 = 0.21$; the crab population increased overwinter, 1970–1971, and decreased the following spring, while shell supplies should have fostered changes in the opposite direction). The data of Figure 4 indicate that under the conditions at Shady Cove, *Pagurus* *granosimanus* numbers will remain constant as long as 0.75–0.80 shells become available for each crab in the population during each month. This is similar to the result of Vance (1972a) and adds further support to his interpretation of his results.

These data support the shell-limitation hypothesis. However, they shed no light upon the actual population processes that lead to the close correspondences observed (Fig. 4). In particular, the constant size structure of the crab population is difficult to reconcile with the large changes in population size. Large changes in population size are usually accompanied by changes in size or age structure (Schaffer and Tamarin, 1973). However, small crabs (in 10–18 mm shells) were not abundant before the population increased, nor were larger crabs more abundant afterwards (Table I). To confirm the hypothesis, studies of actual crab population processes (De Wilde, 1973; Nyblade, 1974) must be combined with those of shell availability.

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SUMMARY

During a six-year period, several thousand intertidal snails, *Thais lamellosa* and *T. emarginata,* were given individually numbered tags and times of death were noted. These snails provided most of the shells used by intertidal hermit crabs (primarily *Pagurus granosimanus*). Therefore changes in the hermit crab population can be related to shell availability. The crab population increased greatly during 1969, and then declined steadily until the end of the study (1973). Changes in the crab population were highly correlated with shell availability. This hermit crab population will remain constant in size if 0.8 shells become available for each crab each month.

LITERATURE CITED

CHILDRESS, J. R., 1972. Behavioral ecology and fitness theory in a tropical hermit crab. *Ecology,* **53**: 960–964.

DRAPKIN, E. I., 1963. Effect of *Rapana bezoar* Linné (Mollusca, Muricidae) on the Black Sea fauna. *Dokl. Akad. Nauk. SSSR Biol. Sci.,* **151**: 964–966.

JENSEN, K., 1975. The profit to *Pagurus bernhardus* (L.) by the presence of *Hydractinia echinata* (Fleming). *Biokon Reports* **1**: 1–4. (Biokon, Prinsesse Maries alle 14, DK-1908, Kobenhavn V. Denmark).

MARKHAM, J. C., 1968. Notes on growth-patterns and shell-utilization of the hermit crab *Pagurus bernhardus* (L.). *Ophelia,* **5**: 189–205.

NYBLADE, C. F., 1974. Coexistence in sympatric hermit crabs. *Ph. D. Thesis, University of Washington,* Seattle, Washington, 241 pp.

PIKE, R. B., AND D. I. WILLIAMSON, 1959. Observations on the distribution and breeding of British hermit crabs and the stone crab (Crustacea: Diogenidae, Paguridae, and Lithodidae). *Proc. Zool. Soc. London,* **132**: 551–567.

Provenzano, A. J., Jr., 1960. Notes on Bermuda hermit crabs (Crustacea; Anomura). *Bull. Mar. Sci.,* **10**: 117–124.

Schaffer, W. M., and R. H. Tamarin, 1972. Changing reproductive rates and population cycles in lemmings and voles. *Evolution,* **27**: 111–124.

Spight, T. M., 1972. Patterns of change in adjacent populations of an intertidal snail, *Thais lamellosa. Ph. D. Thesis, University of Washington,* Seattle, Washington, 308 pp.

Spight, T. M., 1974. Sizes of populations of a marine snail. *Ecology,* **55**: 712–729.

Spight, T. M., 1976. Colors and patterns of an intertidal snail, *Thais lamellosa. Res. Pop. Ecol.,* **17**: 176–190.

Vance, R. R., 1972a. Competition and mechanism of coexistence in three sympatric species of intertidal hermit crabs. *Ecology,* **53**: 1062–1074.

Vance, R. R., 1972b. The role of shell adequacy in behavioral interactions involving hermit crabs. *Ecology,* **53**: 1075–1083.

Wilde, P. A. W. J. De, 1973. On the ecology of *Coenobita clypeatus* in Curaçao. *Stud. Fauna Curaçao,* **44**: 1–138.

Reference: *Biol. Bull.*, **152**: 134–146. (February, 1977)

ACTIVITY AND PHYSIOLOGICAL SIGNIFICANCE OF THE PLEOPODS IN THE RESPIRATION OF *CALLIANASSA CALIFORNIENSIS* (DANA) (CRUSTACEA: THALASSINIDEA)

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The burrowing mud shrimp *Callianassa californiensis* lives in the severely hypoxic environment of the upper (0 to +1 foot) tidal horizon (MacGinitie, 1934). Its respiratory adaptations include a high oxygen affinity hemocyanin (Miller and Van Holde, 1974), a low respiratory rate, a low critical partial pressure, and the ability to tolerate extensive periods of anoxia (Thompson and Pritchard, 1969). Several researchers have stated that the pleopods of *Callianassa* serve a respiratory function by generating water currents within the burrow (MacGinitie, 1934, 1935; Devine, 1966; Farley and Case, 1968). Farley and Case (1968) also presented evidence for a more direct respiratory function when they found that *C. californiensis,* after enduring a period of anoxia, responded to oxygen with a sustained pleopod beat. This suggests that the pleopods are directly involved in gas exchange, perhaps serving a function analogous to that found in isopods where the pleopods account for 50%–74% of total oxygen uptake (Lockwood, 1968). Such a function for the pleopods would be unique among decapod crustaceans.

The present experiments were undertaken to clarify the role of the pleopods in gas exchange in *C. californiensis.*

Materials and Methods

Animals

Animals were collected at Morro Bay State Park, using a shovel, a clam gun, or the "Kiwi Method" which involves jumping on the mud until it liquifies, thereby causing struggling animals to float to the surface where they can be picked up by hand. Animals were placed in fresh sea water and kept in styrofoam coolers for the trip to Santa Barbara. Once in the laboratory, they were kept in mud-filled aquaria supplied with running sea water. Temperatures chosen for each experiment corresponded to the ambient sea water temperature at which the animals were maintained.

The pleopods as a respiratory surface

The contribution of the pleopods to gas exchange was measured by comparing oxygen consumption rates of animals with and without the use of pleopods.

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These rates were determined by allowing individual animals to deplete the oxygen from a Pyrex container filled with filtered sea water and closed to the atmosphere by a lucite lid. Temperature in the glass chamber was maintained at 14.2° C by means of a water jacket connected to a refrigerated water bath. Oxygen partial pressure (PO_2) within the vessel was constantly monitored with a Clark polarographic oxygen electrode (Clark, 1956) as the animal reduced the PO_2 from air saturation to unmeasurably low partial pressures (< 0.1 mm Hg). The time required for this varied from 10 to 14 hours. Streptomycin (60 mg/liter) added to the sea water inhibited bacterial growth. In order to measure the bacterial fraction of total oxygen consumption, after each run the volume of the animal was replaced with air-saturated sea water and oxygen consumption was again measured for 6–12 hours. These rates were subtracted from the total oxygen consumption to yield the consumption due to the animal.

Pleopods were immobilized by glueing them together using cyanoacrylate tissue cement thinly applied to both sides of each appendage. A small piece of polyethylene cut from a plastic bag covered all the pleopods and bound them to the abdomen in the manner of a truss. Animals were held with the thorax in sea water until the glue was dry (5 min) and then were placed in a holding tank for a minimum of 48 hours before being used. The shrimp were starved during this recuperative period. Control animals remained untouched except that they were starved for the same minimum period of time. All animals used were adult males weighing 10–15 g ($N = 9$ per group). A second set of experiments was subsequently run using each individual first as a control and then as an experimental animal, with all other techniques identical to those described above. Animals recovered for 48 hours before the second test. Since each animal was used in both the experimental and control situation, any deviation in respiratory rate could be attributed to the experimental treatment. (This served to control for any sample variance which might lead to misinterpretation of the data.)

Activity measurements

A pleopod beat counter was constructed from an eight inch length of PVC tubing, which had a one inch section around the area of the pleopods partially cut through and bent away, forming two flaps. A miniature photoresistor sealed in epoxy was fastened to one of the flaps. Directly across, on the other flap, a light guide connected to a dissecting lamp was inserted. Each beat of an animal's pleopods crossed the beam of light playing on the photoresistor, unbalancing a wheatstone bridge and generating an electrical pulse. A potentiometric strip chart recorder recorded the pulses.

Animals were placed in the beat counter, lightly restrained with rubber bands, and immediately submerged in a sea water-filled Pyrex vessel. Temperature was controlled at 17.2° C with a constant temperature bath or by continuously flowing sea water through the chamber at the same temperature. In all activity experiments PO_2 was constantly monitored using a Clark-type electrode. All oxygen electrodes were constructed in the laboratory using Clark's design. This gave us the ability to vary cathode diameter and electrode shape to suit a specific experiment. The electrode which was used in both activity and respiration experiments had a cathode diameter of 0.020 inches, yielding an output of one mV at air saturation. This enabled us to monitor the output from the oxygen electrode directly on a one mV chart recorder, eliminating the need for a meter or amplifier. Electrodes were calibrated on the chart recorder using nitrogen and air saturated water. A magnetic stirring bar assured proper mixing in the chamber and sufficient stirring for the electrode to operate properly. The lowest speed possible to satisfy these two criteria was used to minimize any effect on the animal's activity. Pleopod activity was recorded as a function of PO_2 in two different ways. In the first method. the animal itself consumed the oxygen when the chamber was sealed with a lucite lid. In the second, bursts of nitrogen introduced through an airstone lowered the PO_2. No differences were noted in the data collected by the alternate methods. Animals remained in the apparatus for eight hours before experiments were begun.

In order to test the effect of anoxia on pleopod activity, as well as the previously reported oxygen receptor (Farley and Case, 1968), all variables except PO_2 were controlled in the following manner. The chamber and apparatus were the same as described above, but a flowing water system circulated oxygen-depleted water through the Pyrex vessel. Sea water passed consecutively through two columns into which nitrogen was bubbled through an airstone. After water passed through the second column, it entered a bubble trap and then flowed into the chamber. Animals were kept in this apparatus for 14–16 hours before experiments commenced. Activity was recorded for one hour after this period; then air saturated water from the sea water taps flowed through the columns and into the chamber, replacing oxygen depleted water. The animal encountered this sudden influx of air saturated water with no change whatsoever in any of the other ambient physical parameters. No pH differences in the nitrogen and air saturated water were detected.

Behavioral regulation of PO_2

In order to determine how rigorously *C. californiensis* monitored and affected the PO_2 within its immediate microhabitat, a simulated burrow was constructed of plastic tubing. An eight inch section was cut from the middle of a three foot length of 0.75 inch lucite tubing. This small middle section was fitted with a hole large enough to receive the tip of a Clark oxygen electrode. A shrimp could be coaxed into the tubing and kept within by plastic screening fitted over the ends of the small tube. This tube was replaced in the middle of the three foot length by joining the three sections with two tight fitting sleeves of surgical rubber tubing. The resulting apparatus was thus sealed to the outside except at the ends of the three foot length, with the animal confined in the middle. Since the shrimp occupied a small volume (approximately 30 ml), normal oxygen consumption rapidly depleted the PO_2 in the immediately surrounding sea water, forcing the animal to replenish the medium in order to continue normal breathing. Observation indicated that pleopod movement caused fresh sea water (higher PO_2) to flow into the confined animal's vicinity. A microcathode Clark-type oxygen electrode constantly monitored oxygen partial pressure. The diameter of the cathode (0.0006 inch) obviated the need for stirring. All experiments took place in reduced light in a seawater table filled to a depth of four inches. Ten animals were run from 12–72 hours in the apparatus.

pH and PO_2 in the burrows

Water samples were taken at low and high tide from randomly selected burrows of *C. californiensis*. Burrows were inspected for evidence of recent occupation by noting the presence or absence of fecal pellets around the opening to the surface. Stiff 0.25 inch plastic tubing was threaded into the mouth of an occupied burrow to a depth of 18–24 inches. Water was drawn up into a 50 ml syringe, whereupon the plunger was removed and the water in the syringe was tested for pH using pHydrion pH paper (± 0.2 pH units). Oxygen concentration was "frozen" by completing the microwinkler method (Fox and Wingfield, 1938) through the addition of phosphoric acid. As high tide covered the mud flats the syringes floated, anchored by the tubing within the burrow. This allowed sampling to continue through the tidal cycle.

Gill surface area

Gill surface area was measured using a technique modified from Gray (1954, 1957). Filaments of various sizes were removed from several gills, representing one side of the thorax in five freshly killed animals. These were individually traced with a camera lucida. A planimeter was then used to determine the area and length of each filament. In addition, both sides of each gill were likewise traced, with length and area determined in the same manner. The number of filaments were counted for each gill used. Once the above information was obtained, the following calculation determined the gill surface area of the entire animal. A standard curve was constructed of filament length versus filament surface area using the data obtained with the planimeter [$y\ (mm^2) = 0.54 \times (mm) + 0.31$, correlation coefficient = 0.97]. A second standard curve was plotted using total gill length versus number of filaments in the gill. The area of one side of a gill was divided by its length to obtain the average width, or average length filament. The area of the average length filament then could be read off the first standard curve mentioned above. This result, when multiplied by the total number of filaments obtained from the second standard curve, yielded the surface area of one side of the gill. Bilateral symmetry was assumed in dealing with the two sides of the thorax. Thus, values for each complete gill were multiplied by two for each of the ten gills on one side of an animal's thorax to yield the total gill surface area.

RESULTS

The pleopods as a respiratory surface

The mean respiratory rate for animals with and without the use of pleopods was calculated at seventeen partial pressures of oxygen (Fig. 1). The means at each point were compared using a *t*-test for comparison of means with unknown variance not assumed equal (Bailey, 1959). In all seventeen cases the means were not significantly different at the $P > 0.05$ level. Confidence intervals depicted in Figure 1 were calculated using a Student's *t*-distribution and were included to show the variability of the data. The metabolic rate curves were determined by the least squares method. Each group of animals had one line calculated for PO_2 values at 20 mm Hg and above and one for all PO_2 values at 20 mm Hg and

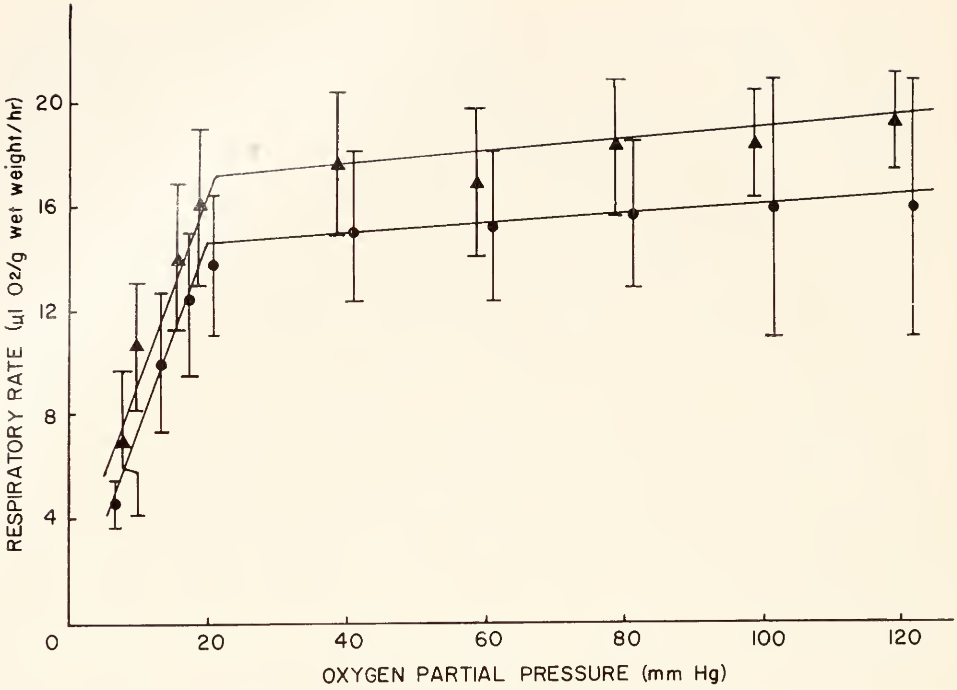

FIGURE 1. Relationship of respiratory rate to oxygen partial pressure in animals with (circle) and without (triangle) the use of pleopods. Error bars indicate 95% confidence limits around each point. Each value is the mean rate of nine animals. Regression lines through the points were calculated using the least squares method. One line was calculated for points 20 mm Hg and above, one line was calculated for points 20 mm Hg and below. The intersection of the lines was called the critical oxygen partial pressure (P_c). Lines were not significantly different. For animals with the use of pleopods, r is 0.86 for the line above 20 mm Hg, and r is 0.94 for values below; mean weight is 12.9 ± 1.3 ($\bar{x} \pm$ s.d.). For animals without the use of pleopods, r is 0.86 for above 20 mm Hg, and r is 0.97 below; mean weight is 12.1 ± 1.7. See text.

below. The intersection of the two lines was called the critical oxygen partial pressure (P_c), the partial pressure of oxygen below which the metabolic rate ceases to be independent of external oxygen concentration (Prosser, 1973). The P_c for control animals was approximately 17 mm Hg, that for animals without the use of pleopods was approximately 20 mm Hg. The variation about the regression lines makes this difference meaningless.

Data collected using individual animals yielded similar results. These respiratory rates were tested using the Wilcoxon test for paired comparisons, at nine PO_2 values above the P_c. Significance levels of the data for the three animals tested were all $>> P = 0.05$, strongly supporting the null hypothesis: treatment and control are the same. It should be mentioned that the metabolic rates obtained in this set of experiments were substantially lower than those reported by Thompson and Pritchard (1969), despite the fact that the temperature at which they were run was 4.2° C higher. This seemingly irreconcilable conflict can perhaps be explained by the fact that the animals used in this study averaged

12.9 ± 1.3 g as compared with 5.3 ± 1.5 g used by Thompson and Pritchard (1969), and were starved for a longer period of time. Since the current study was internally controlled, this conflict does not affect the conclusions drawn from the results.

Activity measurements

The figures in Table I represent pleopod activity levels at three different partial pressures of oxygen, expressed as the percentage of total time active and rate of beat. Since the pleopods of *C. californiensis* beat intermittently, there is variability in both the total time active and the rate of pleopod beat. Intermittent irrigation is a common phenomenon in tubicolous intertidal forms and has been demonstrated in polychaetes (Mangum, 1964), amphipods (Gamble, 1970), stomatopods (Johnson, 1936), and in another species of *Callianassa, C. filholi* (Devine, 1966).

It is interesting to note here that *C. californiensis* varies its percentage of time active, while keeping its rate of beat relatively constant over the PO_2 ranges shown (Table I). Although the amount of time spent in activity is lessened at zero PO_2 when compared with activity at the P_c and above, it is not absent. When in anoxia, animals will struggle when touched. Apparently these shrimp do not enter a true quiescent or lethargic state as was reported by Waterman and Travis (1953) for *Limulus,* and Gray (1957) for *Ocypode,* but do reduce total activity.

A second factor which these data suggest is that these animals do alter their pleopod activity in response to oxygen partial pressure as was reported by Farley and Case (1968). Maximum activity occurs at PO_2 values approaching the P_c. During high tide water of high oxygen concentration is available. Maximal pleopod activity at PO_2 values around the P_c could replace the oxygen depleted water surrounding an animal before aerobic respiration becomes ineffective.

Low tide conditions were simulated in the lab by maintaining the animal at very low oxygen concentrations for 14–16 hours. This produced a different

TABLE I

Relationship of activity to oxygen partial pressure (PO_2) at three PO_2 intervals, expressed as the percentage of total time active and beats per minute (bpm). Values are for individual animals determined over the PO_2 increments shown. Means are not given, due to individual differences.

Animal	PO_2 (mm Hg)					
	100–70		40–15		0	
	Time active (%)	Rate (bpm)	Time active (%)	Rate (bpm)	Time active (%)	Rate (bpm)
1	27.3	26	42.7	22	9.2	18
2	31.9	40	47.9	40	13.7	44
3	77.2	29	77.2	15	28.6	28
4	29.5	14	45.7	15	10.0	15
5	35.2	37	56.7	47	1.0	40

Table II

Initiation of ventilation. Values are for individual animals. Activity is given for animals before and after admission of oxygen (see text).

Before admission of O_2				After admission of O_2			
Animal	PO_2 of system (mm Hg)	Time active (%)	Rate (bpm)	Latency of response (sec)	Time active (%)	Rate (bpm)	Threshold (PO_2 mm Hg)
1	18.6	100	30	20	100	41	27.0
2	22.7	100	26	26	100	50	41.4
3	7.8	0	0	34	100	35	27.3
4	0	40	22	20	100	49	12.0

kind of behavior (Table II). These animals responded to an influx of oxygenated water, analogous to an incoming tide, by sustained rapid beating of the pleopods. Increases in both the percentage of time active (in 50% of the animals) and rate of beat (100% of animals) accompany readmitted oxygen. The difference in quality and quantity of activity between the results of Table I and Table II suggest that long term oxygen deprivation constitutes a stress situation, despite the animal's extensive anaerobic capabilities. In contrast, preliminary experiments with shrimp kept at very low PO_2 for 10–30 minutes gave variable results, often with no response at all to readmitted oxygen.

Behavioral regulation of PO_2

C. californiensis used its pleopods to influence the oxygen partial pressure in the area immediately surrounding it. However, all ten animals observed in this experiment showed only a very coarse regulatory capability (Fig. 2). The data was treated by dividing the total time for each experiment into one half hour increments, and noting the minimum PO_2 within each increment. These incremental minimum PO_2 values were used to obtain a bar graph showing their relative frequency of occurrence during an experiment. Individual runs all showed the same trends; namely, PO_2 dropped to zero on several occasions, sometimes for up to thirty minutes, with the great majority of the total time spent above the P_c. For this reason Figure 2 incorporates all the data obtained, with equal weight given to each run. We feel that this best represents the trends suggested by the data.

This experiment was originally designed to obtain a definite threshold for an oxygen receptor, on the assumption that a shrimp would not permit the oxygen concentration to drop below this point. Since the PO_2 dropped to zero in all cases, it seems that short term exposure to hypoxic conditions causes little or no stress.

pH and PO_2 in the burrows

Table III shows the values obtained for burrow oxygen concentrations and pH at low and high tides. Mean PO_2 varied from 0.59 ± 0.15 ml/liter at low tide to 1.31 ± 0.54 ml/liter at high tide (mean ± s.d.). pH varied from 6.3

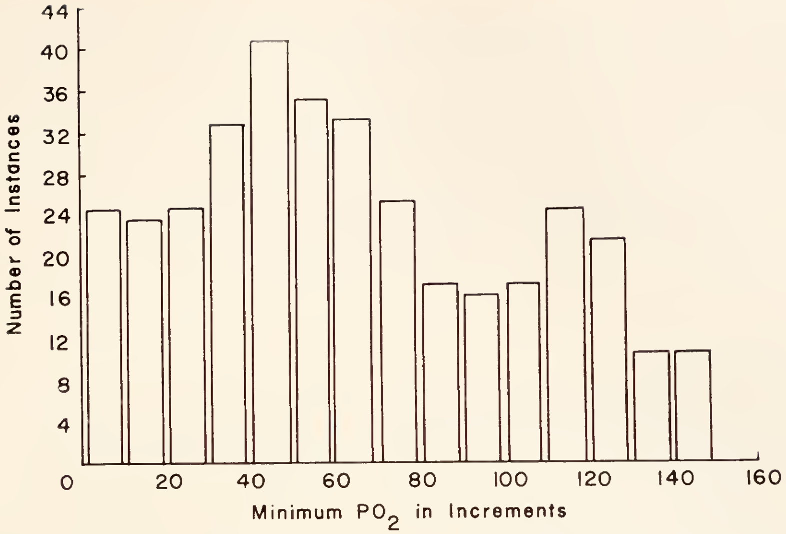

FIGURE 2. Behavioral regulation of PO_2. Abscissa shows the minimum PO_2 found (in a given half hour treatment). Ordinate shows the number of instances a given PO_2 was recorded. Data is from ten animals. See text.

to 7.0. These should be regarded only as approximate values due to the variation in exposure which is caused by tides of different heights. This particular low tide was +0.4 which is relatively high. It caused the burrows to be exposed for approximately 5.5 hours. MacGinitie (1935) reports that *Callianassa* can be exposed for as much as 18 hours in Elkhorn Slough, California, in which case

TABLE III

Oxygen concentration and pH in C. californiensis *burrows at low and high tide. Values are for individual burrow samples. Surface water pH is 7.0; oxygen concentration of surface water is 4.25 ml/liter, temperature, 12.0° C. Means are given with standard deviation.*

Low Tide		High Tide	
O_2 conc (ml/liter)	pH	O_2 conc (ml/liter)	pH
0.60	6.2	0.83	7.0
0.60	6.3	1.55	7.0
0.66	6.4	0.46	7.0
0.23	6.1	1.55	7.0
0.77	6.2	1.20	6.8
0.60	6.4	1.55	7.0
0.66	6.3	1.20	7.0
0.70	7.4	1.13	7.0
0.66	6.2	2.61	7.0
0.60	6.2	1.45	7.0
0.59 ± 0.15	6.30 ± 0.2	1.31 ± 0.54	7.0 ± 0.2

the PO_2 at low tide would certainly drop to zero. The pH would probably be substantially lower as well. Interestingly, immediately after the receding tide exposed the sampled burrows, oxygen concentrations dropped to low tide values. This indicates that even though the burrows of *C. californiensis* at Morro Bay are quite permanent, due to the high clay content of the mud there (Torres, personal observation), there is no barrier to equilibration with interstitial water. Burrows showed no evidence of lining upon close examination. However, in the upper 18 inches they did show a ring of lighter-colored sand about 1 mm in thickness surrounding the burrow walls, indicating a higher PO_2 than the surrounding anaerobic mud.

Gill surface area

Gill surface areas obtained from five shrimp yielded a mean value of 4.13 ± 0.72 cm^2/g wet body weight ($\bar{x} \pm$ s.d.). This figure includes measurements from three adult males and two adult females, ranging from 14.6 mm to 17.0 mm in carapace length, and 4.8 to 6.6 g wet body weight. It is not known whether there is a change in weight specific gill surface area between very small individuals and adults, as has been reported by Belman and Childress (1976) for *Gnathophausia ingens* (Crustacea, Mysidacea), and Gray (1957) for several brachyuran crabs.

DISCUSSION

C. californiensis differs from its congener *C. affinis* and its close relative *Upogebia pugettensis* in that it is not a suspension feeder (MacGinitie, 1930, 1934, 1937, 1939). Rather, *C. californiensis* sifts through the mud in its burrow for detritus. In its constant foraging for food, which includes extending and reworking tunnels, *C. californiensis* often encounters situations of reduced or zero oxygen, even during high tide. Further, due to its high position in the intertidal zone (0 to +1 foot), it is exposed to hypoxia for longer periods of time than most other mud flat dwellers. MacGinitie (1935) reports exposure of these animals for up to 18 hours in Elkhorn Slough, California, and we have observed exposure periods of up to 12 hours at Morro Bay on several occasions.

During low tide exposure, low oxygen and low pH conditions prevail. In this study, burrow water samples contained oxygen concentrations as low as 0.23 ml/liter, with pH values of 6.1 to 6.4. The mean oxygen concentration of 0.59 ± 0.15 ml/liter is equivalent to a PO_2 of 21 mm Hg, closely approaching the P_c of *C. californiensis* (Fig. 1; and Thompson and Pritchard, 1969) and illustrating the fact that even a "high" low tide of +0.4 is sufficient to reduce burrow PO_2 values to a point where aerobic respiration is insufficient.

Callianassa affinis, which inhabits burrows in the rocky intertidal communities of Southern California has to deal with similar low oxygen conditions at low tide. Congleton (1974) reports PO_2 values of 0.5 and 3 mm Hg in samples of burrow water, with interstitial water ranging from 0.8 to 6.1 mm Hg. Neither *C. affinis* nor *C. californiensis* secrete a lining on their burrows and therefore have no barrier to rapid equilibration between burrow and interstitial water at low tide. *Upogebia pugettensis* does possess a tunnel lining, which results in a higher oxygen concentration in its burrow (0.58 ± 0.26 ml/liter) than surrounding interstitial water (0.15 ml/liter) (Thompson and Pritchard, 1969).

Values for burrow oxygen at high tide were surprisingly low, with a mean concentration of 1.31 ± 0.54 ml/liter. These data suggest little interchange between burrow and surface waters when the tide comes in, without active animal intervention. The 0.25 inch tubing used to sample burrows did not prevent water from flowing downward, but it certainly excluded ghost shrimp from the area where it was inserted. It is unlikely that the pleopods of *C. californiensis* can maintain a current strong enough to irrigate its entire extensive burrow habitat, but it is possible that animal movement plays a large part in surface water-burrow interchange, and further local irrigation results in a sufficiently oxygenated immediate microhabitat.

Sustained rapid pleopod beating after long term anoxia (Table II; and Farley and Case, 1968) supports the notion that *C. californiensis* must encourage the flow of surface water into its oxygen-deprived burrow. Several animals were observed in the field forcing water in or out of the burrows directly after being covered by the incoming tide. This is easily discerned by a stream of muddy water flowing from a burrow, or a shifting movement at the mouth of the burrow. These movements were observed in the lab several times and are easily seen when the tide has covered the burrows one to two inchs in depth. Such activity at the surface is closely related to feeding (MacGinitie, 1934), as well as being very possibly involved in respiration. The previously hypothesized function of the oxygen receptor (Farley and Case, 1968) as being a means by which animals might minimize their period of anoxia, thus has added value in signaling the possibility of renewed feeding at the surface.

It is difficult to say what, if any, effects changing pH has on the animal. A preliminary experiment with the effects of CO_2 concentration on activity in one animal showed that high CO_2 concentration, corresponding to a pH of 5.0 induced quiescence, but anything closer to 7.0 had no noticeable effect. It may be that external pH at the levels found in the burrow has little effect on *C. californiensis*.

C. californiensis feeding behavior must be included in any consideration of the animal's relationship to its environment. Since it is a deposit feeder, it often constructs new tunnels (Peterson, 1972), making numerous encounters with hypoxia unavoidable. Some of these encounters may be minimized by irrigation with the pleopods, since the animal does respond to lowered oxygen with increased pleopod activity (Table I; and Farley and Case, 1968). However, when building a new tunnel, there will often be no oxygenated water available for circulation. In these situations *C. californiensis* may continue to function anaerobically to satisfy its energy needs. Short periods of anaerobiosis do not seem to tax this animal, as was observed both in preliminary experiments on oxygen perception and in the behavioral PO_2 regulation experiments (Fig. 2). It is not surprising that ghost shrimp may sometimes rely on anaerobiosis for energy, for hypoxic conditions would otherwise severely restrict the animal's movements, even during high tide.

Long periods of anoxia, such as that produced by minus tides, warrant an eventual reduction in total activity (Table I). A similar response to declining oxygen (preliminary hyperactivity, followed by quiescent behavior), has also been noted in *Typhlogobius californiensis* (Teleostei: Gobiidae) (Congleton, 1974). *Typhlogobius* is a commensal in the burrows of *Callianassa affinis,* and is subject to the same types of respiratory stress as *C. californiensis.* In both cases, minimal activity during prolonged anoxia may serve to reduce oxygen debt.

The respiratory adaptations of *Typhlogobius* and *C. californiensis* are very similar despite their widely different phyletic origins. A 1.4 g *Typhlogobius* has a respiratory rate of 21 μl O_2/hr at 15° C (Congleton, 1970), as compared with a rate of $\sim$ 15 μl O_2/g wet weight/hr in *C. californiensis* at 14.2° C. Oxygen consumption in *Typhlogobius* is much lower than that reported for *C. californiensis* by Thompson and Pritchard (1969); however both this study and that of Congleton used animals in a postabsorptive state of starvation, with experiments conducted at a similar temperature, allowing for better comparison. Further similarities include a similar P_c (9–16 mm Hg for *Typhlogobius;* 10–20 mm Hg for *C. californiensis*), and a large anaerobic capacity ($\sim$ 4 days for *T. californiensis;* 5.7 days for *C. californiensis*).

The characteristics of aerobic respiration in both *C. californiensis* and *Typhlogobius* are probably indicative of adaptations to high rather than low tide oxygen conditions. A low respiratory rate is useful in conserving what limited oxygen is available in a burrow; a low P_c allows aerobic respiration to continue in low oxygen areas within the burrow at high tide. After exposure, extremely low oxygen concentrations preclude the possibility of aerobic respiration. It is for this reason that a highly efficient system of oxygen extraction is not needed. Oxygen is either available, or it is not, depending on the tidal position.

C. californiensis shows no extrabranchial oxygen exchange across the pleopods, which on inspection would be ideally suited for this purpose (Fig. 2). These appendages have a large hemocoel, as well as a large surface area, and would add considerably to the gas exchange surface of the animal. The fact that they serve mainly in propelling water may indicate that additional respiratory surface is unnecessary for survival. It is corroborated to some extent by the low gill surface areas of *Callianassa* (4.13 cm^2/g). This value is lower than any yet reported in the literature for a totally aquatic crustacean (Gray, 1957; Hughes, Knights, and Scammel, 1969).

A large gill surface area and a highly efficient system of oxygen extraction are more typical of either very active Crustacea such as the portunids (Gray, 1957), or animals from a stable low oxygen environment such as *Gnathophausia ingens,* the oxygen minimum layer mysid (Childress, 1971; Belman and Childress, 1976), or *Pleuronocodes planipes,* the galatheid red crab (Quetin and Childress, 1976). Oxygen concentrations as low as 0.20 ml/liter are typical of oceanic oxygen minimum layers (Schmidt, 1925; Sewell and Fage, 1948; Banse, 1964). However, many crustaceans which inhabit zones of oxygen minimum are believed to live entirely aerobically (Childress, 1971; 1975) and often have little or no capability for anaerobiosis (Childress, 1975; Torres, unpublished data).

Total dependence on the limited oxygen available for respiration is allowed for by the stability of an oxygen minimum layer. In contrast, the relative instability of the high intertidal environment of *Callianassa* would preclude anything but minimum dependence on environmental oxygen, that is, facultative anaerobiosis.

We wish to thank Z. M. Fuzessery, L. B. Quetin, Dr. A. W. Ebeling and Dr. A. M. Wenner for critically reviewing the manuscript. We also thank Janet K. Larsen for her help in all phases of this study. This study was supported by the National Science Foundation (OCE-7201413) and (OCE76-10407).

SUMMARY

1. The pleopods of *C. californiensis,* a potential site for extrabranchial oxygen exchange, do not contribute significantly to oxygen consumption.

2. *C. californiensis* has a gill surface area of 4.13 ± 0.72 cm^2/g wet body weight, the lowest value yet reported for a totally aquatic crustacean.

3. *C. californiensis,* when placed in simulated burrow conditions, regulates the PO_2 very loosely in its immediate microhabitat, using its pleopods.

4. Field studies of pH and PO_2 values in burrows of *C. californiensis* indicate that animal movement may play a large part in water exchange between the surface and burrow.

5. Activity studies suggest that oxygen is not critical to *C. californiensis* on a short term basis. Perception of oxygen after long deprivation may signal the possibility of renewed feeding and activity at the surface of its burrow.

LITERATURE CITED

BAILEY, N. T. J., 1959. *Statistical methods in biology.* English Universities Press, London, 200 pp.

BANSE, K., 1964. On the vertical distribution of zooplankton in the sea. Pages 53–125 in M. Sears, Ed., *Progress in oceanography, Volume II.* Pergamon Press, Oxford.

BELMAN, B. W., AND J. J. CHILDRESS, 1976. Circulatory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens. Biol. Bull.,* **150**: 15–37.

CHILDRESS, J. J., 1971. Respiratory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens. Biol. Bull.,* **141**: 109–121.

CHILDRESS, J. J., 1975. The respiratory rates of midwater crustaceans as a function of depth of occurrence and relation to the oxygen minimum layer off Southern California. *Comp. Biochem. Physiol.,* **50A**: 787–799.

CLARK, L. C., 1956. Monitor and control of blood and tissue oxygen tensions. *Trans. Amer. Soc. Artificial Internal Organs,* **2**: 41–48.

CONGLETON, J. L., 1970. Response to asphyxia of *Typhlogobius californiensis* Steindachner and some related gobies. *Ph.D. thesis, University of California, San Diego,* 132 pp. (*Diss. Abstr.,* **31**: 5314B; order no. 71–4228.)

CONGLETON, J. L., 1974. The respiratory response to asphyxia of *Typhlogobius californiensis* (Teleostei: Gobiidae) and some related gobies. *Biol. Bull.,* **146**: 186–205.

DEVINE, C. E., 1966. Ecology of *Callianassa filholi* Milne-Edwards 1878 (Crustacea, Thalassinidea). *Trans. Roy. Soc. N. Z. Zool.,* **8**: 93–110.

FARLEY, R. D., AND J. F. CASE, 1968. Perception of external oxygen by the burrowing shrimp *Callianassa californiensis* Dana and *C. affinis* Dana. *Biol. Bull.,* **134**: 361–365.

FOX, H. M., AND C. A. WINGFIELD, 1938. A portable apparatus for the determination of oxygen dissolved in a small volume of sea water. *J. Exp. Biol.,* **15**: 437–445.

GAMBLE, L. C., 1970. Effect of low dissolved oxygen concentrations on the ventilation rhythms of three tubicolous crustaceans, with special reference to the phenomenon of intermittent ventilation. *Mar. Biol.,* **6**: 121–127.

GRAY, I. E., 1954. A comparative study of the gill area of marine fishes. *Biol. Bull.,* **107**: 210–225.

GRAY, I. E., 1957. A comparative study of the gill area of crabs. *Biol. Bull.,* **112**: 34–42.

HUGHES, G. M., B. KNIGHTS, AND C. A. SCAMMEL, 1969. The distribution of PO_2 and hydrostatic pressure changes within the branchial chambers in relation to gill ventilation of the shore crab *Carcinus maenas* L. *J. Exp. Biol.,* **51**: 203–220.

JOHNSON, M. L., 1936. The control of respiratory movements in crustacea by oxygen and carbon dioxide. II. *J. Exp. Biol.*, **13**: 467–475.

LOCKWOOD, A. P. M., 1968. *Aspects of the physiology of Crustacea.* Oliver and Boyd, London, 328 pp.

MACGINITIE, G. E., 1930. Natural history of the mud shrimp *Upogebia pugettensis* (Dana). *Amer. Midland Natur.*, **6**: 36–44.

MACGINITIE, G. E., 1934. The natural history of *Callianassa californiensis* Dana. *Amer. Midland Natur.*, **15**: 166–177.

MACGINITIE, G. E., 1935. Ecological aspects of a California marine estuary. *Amer. Midland Natur.*, **16**: 629–765.

MACGINITIE, G. E., 1937. Notes on the natural history of several marine crustacea. *Amer. Midland Natur.*, **18**: 1031–1037.

MACGINITIE, G. E., 1939. The natural history of the blind goby, *Typhlogobius californiensis* Steindachner. *Amer. Midland Natur.*, **21**: 489–505.

MANGUM, C. P., 1964. Activity patterns in metabolism and ecology of polychaetes. *Comp. Biochem. Physiol.*, **11**: 239–256.

MILLER, K., AND K. E. VANHOLDE, 1974. Oxygen binding by *Callianassa californiensis* hemocyanin. *Biochem. J.*, **13**: 1668–1674.

PETERSON, C. H., 1972. Species diversity, disturbance, and time in the bivalve communities of some California lagoons. *Ph.D. Thesis, University of California, Santa Barbara,* 241 pp. (*Diss. Abstr.*, **33**: 2030B; order no. 72–26,836.)

PROSSER, C. L., 1973. *Comparative animal physiology.* W. B. Saunders, Co., Philadelphia, 955 pp.

QUETIN, L. B., AND J. J. CHILDRESS, 1976. Respiratory adaptations of *Pleuroncodes planipes* Stimpson to its environment off Baja California. *Mar. Biol.*, in press.

SCHMIDT, J., 1925. On the contents of oxygen in the ocean on both sides of Panama. *Science,* **61**: 582–583.

SEWELL, R. B. S., AND L. FAGE, 1948. Minimum oxygen layer in the ocean. *Nature,* **162**: 949–951.

THOMPSON, R. K., AND A. W. PRITCHARD, 1969. Respiratory adaptations of two burrowing crustaceans, *Callianassa californiensis* and *Upogebia pugettensis. Biol. Bull.*, **136**: 274–287.

WATERMAN, T. H., AND D. F. TRAVIS, 1953. Respiratory reflexes and the flabellum of *Limulus. J. Cell. Comp. Physiol.*, **41**: 261–289.

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CONTENTS

BLACKWELL, JOAN F., LOUIS F. GAINEY, JR., AND MICHAEL J. GREENBERG
Shell ultrastructure in two subspecies of the ribbed mussel, *Geukensia demissa* (Dillwyn, 1817) 1

BREHM, PAUL AND JAMES G. MORIN
Localization and characterization of luminescent cells in *Ophiopsila californica* and *Amphipholis squamata* (Echinodermata: Ophiuroidea) 12

HAYES, RAYMOND L. AND NORA I. GOREAU
Intracellular crystal-bearing vesicles in the epidermis of scleractinian corals, *Astrangia danae* (Agassiz) and *Porites porites* (Pallas) 26

HEDGECOCK, DENNIS, KEITH NELSON, JAMES SIMONS, AND ROBERT SHLESER
Genic similarity of American and European species of the lobster *Homarus* 41

HENDLER, GORDON
Development of *Amphioplus abditus* (Verrill) (Echinodermata: Ophiuroidea): I. Larval biology 51

HORI, SAMUEL H., KUNIO TANAHASHI AND NORIMASA MATSUOKA
Morphological and cytochemical studies on the secretary granules of the pyloric caeca of the starfish, *Asterias amurensis* 64

LANG, FRED, WALTER J. COSTELLO, AND C. K. GOVIND
Development of the dimorphic claw closer muscles of the lobster *Homarus americanus:* I. Regional distribution of muscle fiber types in adults 75

LANG, W. H. AND A. M. YOUNG
The larval development of *Clibanarius vittatus* (Bosc) (Crustacea: Decapoda; Diogenidae) reared in the laboratory 84

MOORE, CAROL A. AND ALBERT F. EBLE
Cytochemical aspects of *Mercenaria mercenaria* hemocytes 105

SPIGHT, TOM M.
Availability and use of shells by intertidal hermit crabs 120

TORRES, J. J., D. L. GLUCK, AND J. J. CHILDRESS
Activity and physiological significance of the pleopods in the respiration of *Callianassa californiensis* (Dana) (Crustacea: Thalassinidea) 134

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Continued on Cover Three

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THE

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INTERACTION BETWEEN PHOTOPERIOD, TEMPERATURE, AND CHILLING IN DORMANT LARVAE OF THE TREE-HOLE MOSQUITO, *TOXORHYNCHITES RUTILUS* COQ.

Reference: *Biol. Bull.*, **152**: 147–158. (April, 1977)

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Many temperate zone insects overwinter in a state of dormancy or diapause, where development ceases but other physiological and behavioral processes may remain active. For a great many insects, diapause may be terminated after prolonged chilling (Andrewartha, 1952; Lees, 1955; Williams, 1956) or by photoperiod (Lees, 1968; Danilevskii, 1965; Beck, 1968; Danilevskii, Goryshin, and Tyshchenko, 1970; Tauber and Tauber, 1976a). Even when photoperiod is important for the initiation and maintenance of dormancy, low temperatures may terminate diapause so that resumed development is independent of photoperiod (Danilevskii, *et al.*, 1970; Tauber and Tauber, 1976a). The two processes, response to day length and to chilling, are usually visualized as distinct, even though Bradshaw (1974) and Tauber and Tauber (1975) have shown that the critical photoperiod as well as the depth of dormancy may change during diapause. Neither of these studies clearly distinguished between the effects of low temperatures and those of prolonged exposure to short-day photoperiod.

The carnivorous tree-hole mosquito, *Toxorhynchites rutilus,* overwinters as a terminal (fourth) instar within the rot holes of deciduous trees in eastern North America (Holzapfel and Bradshaw, 1976). In Pennsylvania, the critical photoperiod and depth of diapause are considerably lower among larvae captured in January than among those caught in September or reared in the laboratory (Bradshaw and Holzapfel, 1975). These differences in the dormant state of individuals may be due to exposure to short days, to low temperatures, or to both. This study attempts to resolve to what extent the maintenance and depth of diapause is dependent upon photoperiod, temperature, and chilling.

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MATERIALS AND METHODS

All animals in the present study belong to the F_1, F_2, or F_3 generation of *Toxorhynchites rutilus septentrionalis* from Lahaska, Bucks County, Pennsylvania, 40°20′N. latitude, 75°04′W. longitude, and 180 m altitude. The P_1 generation was caught as diapausing larvae during September, 1973, and the subsequent colony maintained by induced mating (Trimble and Corbet, 1975; Holzapfel and Bradshaw, 1976). To initiate diapause, the larvae were reared under short-day conditions at 25 ± 0.5° C (9L:15D); they were maintained and experimentally used in individual 60 × 15 mm petri dishes. First and second instar larvae received freshly hatched brine shrimp (*Artemia salina*) as food; second and third instars received *Tubifex* worms. The larvae were fed, cleaned, and observed daily. To obtain a uniform cohort of individuals for the chilling experiment, only F_2 larvae which had hatched over an eleven day period and had spent 9–17 days as diapausing fourth instar larvae prior to the initiation of the experiments were used.

Illuminations included a single 15W tungsten lamp at a distance of 10–15 cm at 4 ± 2 C° and 7 ± 1° C, two 40W cool white fluorescent lamps at a distance of 1.5 m at 16.5 ± 1° C, and a single 4W cool white fluorescent lamp at a distance of 10–15 cm at 16.5 ± 1, 21 ± 1, and 25 ± 1° C.

Tree-hole and air temperatures were recorded by a Rustrak Model 2133 thermistor-probe recorder, equipped with a time-sharing feature to record two temperatures at the same time.

RESULTS

Development of unchilled animals at three constant temperatures

The effects of constant temperature on the development of unchilled animals were examined by comparing photoperiodic response and rates of development among larvae at 25, 21, and 16.5° C. Nine to fourteen larvae were exposed at each temperature and eight photoperiods, allowing 40 days for development at 25° C, 50 days at 21° C, and 80 days at 16.5° C. Figure 1A shows that the critical photoperiods were similar at all three temperatures, ranging from 12.9 hours at 25° C to 13.2 hours at 21° C with an intermediate value, 13.1 hours at 16.5° C. None of the larvae developed in response to 12 hours of light per day at any temperature; all of the larvae at each temperature developed within the experimental period when they were exposed to 14 or more hours of light per day. To obtain linear distributions from the sigmoid distributions shown in Figure 1A, percentage data were transformed first by setting values of 100 or 0 per cent equal to 99.99 or 0.01 per cent, respectively; then, $\%\ (\text{transformed}) = \ln\left[(100 - \%)/\%\right]$. Regression of the transformed percentage of development on photoperiod and temperature indicated that development was significantly correlated with photoperiod ($F = 47.81$; $P < 0.001$) but not temperature ($F = 0.97$; $P > 0.3$), the former explaining 77% of the variation in the percentage of development, the latter less than 2%. Thus, temperature had little or no role in the termination of diapause, *per se,* among unchilled individuals of *T. rutilus.*

Figure 1B shows the rates of development at the two photoperiods (14L:10D and 13L:11D) for which data were available at all three temperatures. Two-

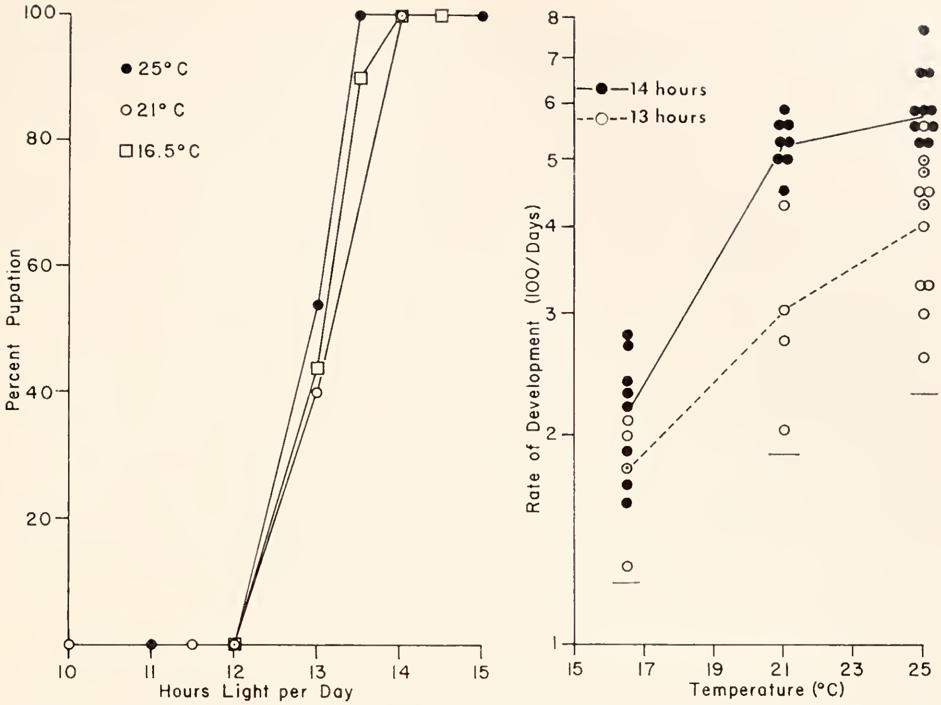

FIGURE 1. Effects of temperature and photoperiod on development. Left-hand graphs (A) show percentage of development in response to various photoperiods at 25°, 21°, and 16.5° C. Only photoperiod has a significant effect on percentage development. Right-hand plots (B) show rate of development in response to long (14 hour) and intermediate (13 hour) photophases at 25°, 21°, and 16.5° C. The horizontal line at each temperature shows the time interval of the experiment, *i.e.*, the minimum rate that could be observed at each temperature. Within this time interval, both temperature and photoperiod have a significant effect on rates of development.

way analysis of variance for unequal but proportional subclasses was carried out according to Sokal and Rholf (1969), after logarithmic transformation to achieve homogeneity of variance. Where subclasses were not proportional, individuals were eliminated by assigning each a number and excluding those numbers indicated by turning cards. Two-way analysis of variance revealed significant effects of both photoperiod ($F_{1,\,31,} = 7.76$; $P < 0.01$) and temperature ($F_{2,\,31,} = 16.23$; $P < 0.01$) and no significant interaction between temperature and photoperiod ($F_{2,\,31} = 2.92$; $P > 0.05$). Thus, among unchilled larvae, there was a quantitative and additive effect of both photoperiod and temperature on the rate of development.

Chilling at four constant temperatures

The effects of prolonged exposure to low temperature were examined by placing 23 larvae each on a short-day regimen (9L:15D) at 4 ± 2°, 7 ± 1°, 16.5 ± 1°, or 21 ± 1° C for 159 days. During this time, only 3 out of 23 larvae survived at 4° C, while 19–21 survived at the higher temperatures (Table IA). The surviving

TABLE 1

Effects of chilling for 159 days at various temperatures followed by long (15L:9D) or short (9L:15D) days at 21° C.

	Chilling temperature			
	4° C	7° C	16.5° C	21° C
A. Per cent surviving 159 days chilling	13 (23)*	91 (23)	87 (23)	83 (23)
B. Per cent surviving 50 short days at 21° C after chilling	67 (3)	73 (11)	100 (11)	64 (11)
C. Per cent of survivors which develop within 50 short days at 21° C	0 (2)	38 (8)	0 (11)	0 (7)
D. Per cent larval survivorship on long days at 21° C after chilling	—	70 (10)	90 (10)	90 (9)
E. Rate of development on long days at 21° C after chilling	—	10.05 ± 2.12 (7)**	6.33 ± 1.18 (9)	5.26 ± 1.29 (8)

* Sample size is given in parentheses.
** Mean plus or minus two standard errors.

larvae were placed on long (15L:9D) or short (9L:15D) days at 21 ± 1° C. Of those exposed to the short-day regimen, 64–100% survived for at least 50 days, but only those which had been chilled at 7° C exhibited any development (Table IB–C). All of the larvae on the long-day regimen had either developed or died within 50 days. Larval survivorship ranged from 70–90% (Table ID), and mean rates of development (100/days to molt to a pupa) ranged from 5.26 to 10.50 (Table IE). Analysis of variance indicated significant differences between means ($F = 22.26$; $P < 0.01$). Duncan's multiple range test showed highly significant differences between the rate of development among larvae chilled at 7° C and those chilled at either 16.5° or 21° C. The difference in mean rates between larvae chilled at the latter two temperatures was not significant. These results indicated that prolonged exposure to 4° C produced substantial mortality and that the chilling "optimum" in terms of diapause termination or accelerated response to long-day photoperiod was above 4° C and below 16.5° C.

Effects of chilling on critical photoperiod

The effect of chilling on qualitative (critical photoperiod) as well as quantitative (rate) measures of development was assessed by chilling larvae at 7° C for up to six months and then by exposing them to various photoperiods at 21° C. A uniform cohort of 350 individuals of *T. rutilus* was reared at 21 ± 1° C, with nine hours of daily illumination. After 9–17 days as fourth instar larvae, 300 individuals were placed at 7 ± 1° C with a 9L:15D photoperiod, and ten larvae were exposed to each of the following photophases per 24 hour day: 10, 11.5, 12, 13, and 14 hours at 21 ± 1° C. At 45 day intervals thereafter, 50 larvae were removed from the colder temperature and were subjected in samples of 10 each to various photophases at 21° C; photophases were determined from the results of ongoing experiments. After 180 days of chilling, samples of 14 larvae each were used to compensate for expected mortality.

Figure 2 shows that the critical photoperiod for development within 50 days of transfer to 21° C decreased consistently with prolonged chilling at 7° C. Prior to chilling, the critical photoperiod was 13.20 hours of light per day; after six months chilling, it had declined to 11.45 hours. In addition, after 180 days chilling, a substantial proportion of larvae developed in response to 9 or 10 hours of light per day.

The effects of chilling were distinguished from those of prolonged exposure to short days by comparing developmental response of unchilled individuals of *T. rutilus* with that of larvae chilled for six months after a total experimental time of 230 days (230 days at 21° C *vs.* 180 days at 7° C, plus 50 days at 21° C). After 230 days at 21° C (Fig. 2), the critical photoperiod for unchilled larvae was one hour longer than that of the animals chilled for six months and none of the larvae exposed to 10 hours of light per day had yet developed. These results showed that chilling, independent of short-day photoperiod, had a quantitative effect on the critical photoperiod and, after prolonged exposure, may have terminated diapause directly.

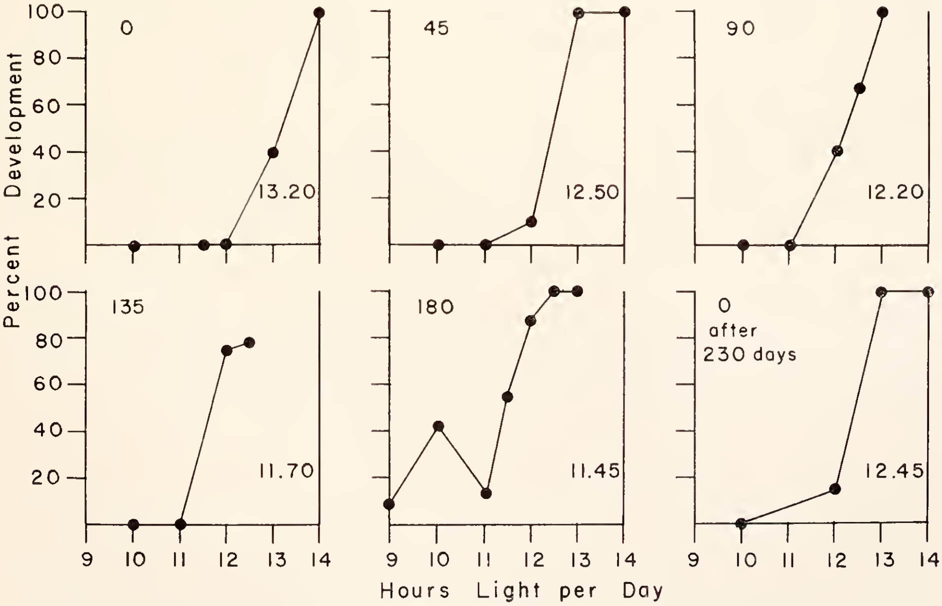

FIGURE 2. Qualitative effect of chilling on the termination of diapause. Larvae were chilled at 7° C for the number of days shown in the upper-left corner of each graph and then exposed to various photoperiods for 50 days at 21° C. Development was scored by noting the percentage of larvae which molted to pupae. Critical photoperiods are given in the lower right corner of each graph.

Effects of chilling on rates of development

The results in Figure 1A and Figure 2 showed that at photoperiods intermediate between long and short, an intermediate percentage of the sample population developed. These results dealt with development at the population level, and the question remained as to whether the population-statistical results were indicative of the response by individual larvae. Development through to pupation was an all-or-none response, but the rate at which each individual responded reflected the depth of its diapause and its physiological interpretation of environmental cues. Prior to chilling (Fig. 3A), 14 hours of light per day elicited a uniformly rapid rate of development and 13 hours, a more various and intermediate rate of development. With prolonged chilling, rates of development in response to the longest photoperiods progressively increased (Fig. 3F), as did the variability in response to intermediate daylengths (Fig. 3C–D). After 180 days chilling (Fig. 3E), rates of development separated into two distinct groups: those which molted to pupae within 15 days (at a rate of 6.7 or higher) and those which did not molt for 150 days (at a rate of 0.67). Among those larvae which molted within 150 days of transfer to 21° C (Fig. 3E), there was no significant difference in the rates at any photoperiod (analysis of variance: $F = 2.04$; $P > 0.05$). Prior to chilling, the mean rate of development in response to long days was 5.25 ± 0.25 (Fig. 3F) and after 159 additional short days at 21° C was still 5.26 ± 1.29 (Table IE). By marked contrast, there was a steady increase in the rate of development among the animals experiencing short days at 7° C and subsequently exposed to long days at 21° C (Fig. 3F). Finally, after 6 months at 7° C, some larvae developed in response to 9 and 10 hour photophases; yet, none of the larvae having experienced a 10 hour photophase continuously at 21° C developed for over 230 days (Fig. 2).

These results show that with progressive chilling, individuals lost their graded response to photoperiod and no longer interpreted any photoperiods as intermediate but only as long or short. At the same time, prolonged chilling decreased the depth of diapause and eventually effected its termination.

Chilling in *T. rutilus* plays a triple role. First, chilling can promote a response to progressively shorter daylengths, thus decreasing the critical photoperiod. Secondly, chilling can accelerate response to long days, thereby decreasing the depth of diapause. Thirdly, prolonged chilling can eventually terminate diapause directly, leaving subsequent morphogenesis independent of photoperiod.

Mortality after prolonged chilling

Mortality was low during chilling at 7° C (Table I). Mortality among unchilled larvae remained low for about 150 days, after which it increased sharply, reaching 36% by the end of the experiment (344 days) (Fig. 4). No appreciable difference in the pattern was observed among larvae chilled for 45, 90, or 135 days. Among larvae chilled for 180 days, there was an initial burst in mortality, which reached 30% within 30 days. By the end of the experiment, mortalities ranged from 36 to 40% regardless of chilling time.

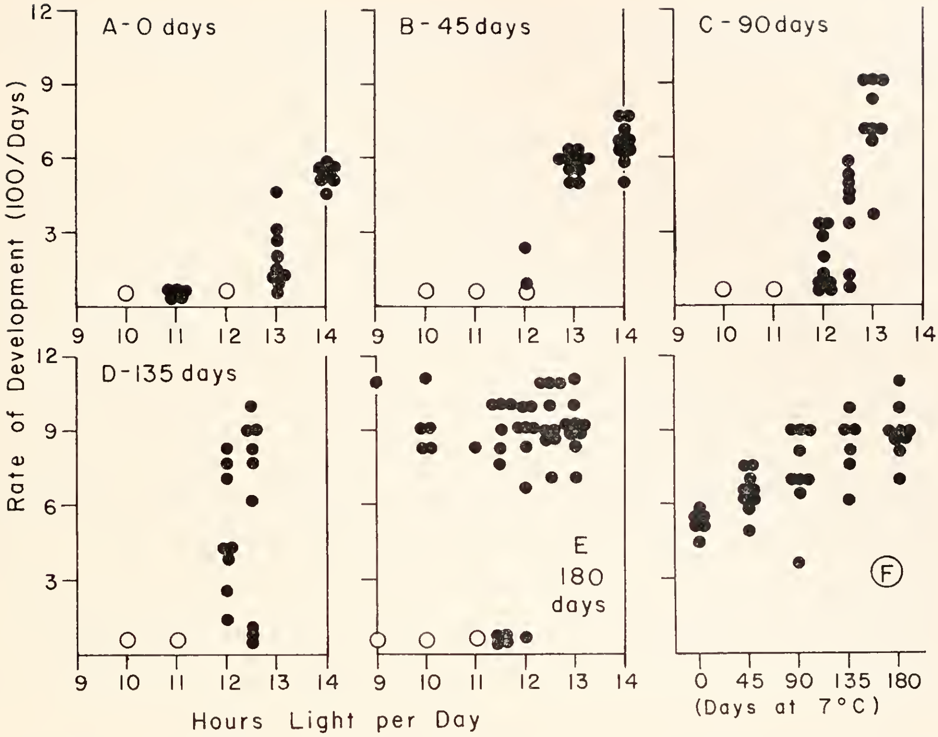

FIGURE 3. Quantitative effect of chilling and photoperiod on rates of development. Larvae and conditions are the same as in Figure 2. A–E show rates of development at various photoperiods after different chilling times; F, rates of development greater than 1.0 (100 days to molt to a pupa) at the longest photoperiod in A–E after different chilling times. Solid circles represent individuals; open circles represent six or more individuals.

Tree-hole temperature

To obtain tree-hole temperatures in nature and to correlate these temperatures with local ambient temperatures, a tree-hole was selected near Lahaska, Pa. Larvae of *T. rutilus* had been found in this hole for four consecutive winters, and large numbers of eggs had been observed there during the summer months. The hole lay about 4.2 m above the ground in an exposed maple tree and contained up to 5 liters of water. One probe of the recorder was placed in the water of the rot-hole, the other outside of the hole, about 1 cm from the bark on a shaded portion of the tree, 4.3 m from the ground. Temperatures were obtained continuously from May 14–20 and May 28 to June 15, 1976. During this period, air temperatures ranged from 3.0° to 30.0° C and tree-hole temperatures from 9.0° to 21.0° C. The temperature cycle in the tree-hole was of a lower amplitude and lagged that of the air. There was a close correlation ($R = 0.93$; $F = 69.7$; $P < 0.001$) between mean tree-hole temperature, TTH, and the weighted means of air temperature during the current, TA_t, and previous, TA_{t-1}, days: $TTH = 5.95 + 0.412TA_{t-1} + 0.188TA_t$. For practical purposes, the formula can be simplified to the form

$TTH = 6 + (\frac{1}{5})(TA_t + 2TA_{t-1})$, with no decrease in the coefficient of multiple correlation ($R = 0.93$) (Fig. 5A). Therefore, at least during the month of May and June, tree-hole temperature was closely correlated with air temperature.

DISCUSSION

Prior to chilling, larval diapause in *T. rutilus* is maintained primarily by photoperiod (Figs. 1A and 2; Bradshaw and Holzapfel, 1975; McCrary, 1965). The critical photoperiod of this response varies as little as plus or minus 7.5 minutes over the range of 16.5° to 25° C (Fig. 1A). Moreover, the slopes of the lines between threshold and saturation do not appear to differ over these temperatures. The photoperiodic mechanism is thus highly temperature-compensated, maintaining a given set-point (critical photoperiod) and inherent accuracy (slope of the response curve) between 16.5° and 25° C. Although the photoperiodic clock imposes an all-or-none response on each individual over a wide range of temperatures, both temperature and photoperiod affect the rate of ensuing development (Fig. 1B). These differences in rates of development at various temperatures and photoperiods could be due to either variation in the rate of termination of diapause, *per se*, or in the completion of post-diapause morphogenesis. Regardless of the

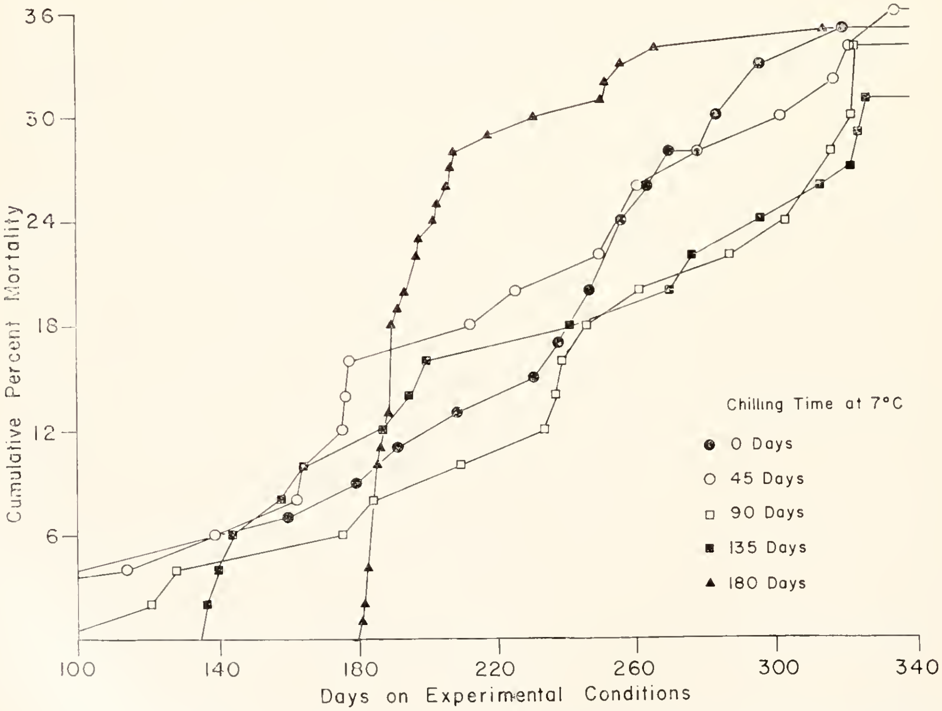

FIGURE 4. Mortality of larvae at 21° C (all photoperiods combined) after various chilling times. Days on experimental conditions refer to days since the start of the initial chilling and do not include rearing times up to the fourth instar.

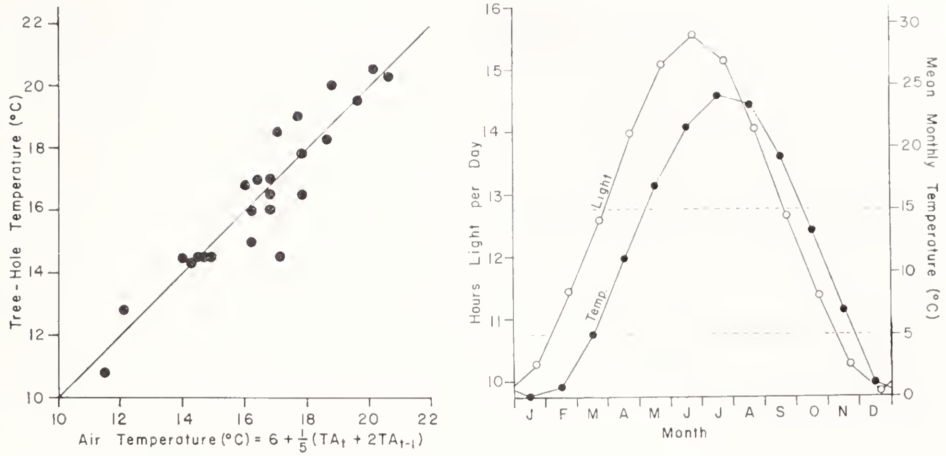

FIGURE 5. Light and temperature relationships near Lahaska, Pennsylvania. Left-hand plot (A) shows relationship between mean tree-hole temperature and air temperature. TA_t and TA_{t-1} are mean daily air temperatures of the current and previous day, respectively. Mean daily temperatures are calculated as the average of the daily maximum and minimum. Right-hand curves (B) show the seasonal march of both temperature (94-year average) and light (sunrise to sunset plus one civil twilight) for Philadelphia, Pennsylvania. Temeperatures were taken from the *Climatic Atlas of the United States* (United States Department of Commerce, 1968) and daylenghs from *Tables of Sunrise, Sunset, and Twilight* (Nautical Almanac Office, United States Naval Observatory, 1962).

mechanisms involved, an individual committed to resumed development by virtue of its photoperiodic clock will assume a more conservative rate at lower temperatures and photoperiods than at higher or longer ones.

In terms of rates of development (Fig. 3), chilling increases the responsiveness to all photoperiods. Thus, increased responsiveness to longer days results in a decrease in the depth of diapause; increased responsiveness to shorter and intermediate days results in a decrease in the critical photoperiod. Short of evoking the termination of diapause directly, these effects of chilling remain hidden until the larvae subsequently experience photoperiods at temperatures compatible with morphogenesis. It is tempting to draw a parallel between the accumulated facilitation of development in *T. rutilus* and the summation of covert effects of ecdysone in the fleshfly, *Sarcophaga peregrina* (Ohtaki, Milkman, and Williams, 1968). In *S. peregrina,* ecdysone is rapidly inactivated, but its effects accumulate so that even though the titer of ecdysone at any one time remains low, the accumulated effects render the target tissues sensitive to progressively less additional hormone. Analogously, in *T. rutilus,* chilling temperatures may cease to prevail, but the summation of their covert effects remains. Relative to unchilled larvae, fewer days additional chilling or shorter daylengths are then required to terminate diapause. Since both chilling and photoperiod may affect the brain directly in other insects (Williams, 1956; Williams and Adkisson, 1964), the covert effects of these same factors may summate in the brain of *T. rutilus*, rendering it more responsive to subsequent stimulation or, eventually, terminating diapause.

The termination of diapause after prolonged chilling may serve as a safety valve, since this role is expressed only when the larvae are approaching the limits of tolerance to long exposure at low temperature (Fig. 4). The adaptive significance of the interaction between temperature and photoperiod is not so apparent. As shown in Table I, chilling has its greatest effect above 4° and below 16.5° C. Even though there is a significant chilling effect at 7° C and a substantial reduction of the critical photoperiod produced by the same temperature (Figs. 2 and 3), the optimum may be somewhat higher. Figure 5B shows mean monthly temperature for Philadelphia, Pennsylvania, approximately 60 km from Lahaska. Figure 5A shows that the tree-hole temperature is closely correlated with air temperature during May and June. Although tree-hole and air temperature data for all seasons were not obtained, direct observation revealed that the tree-hole from which the recordings were made froze over for most or all of January, indicating that temperatures of 4° C or lower prevail at this time of the year. If 5–15° C may be taken as the range of temperatures which are likely to be important for promoting the chilling reaction, it is apparent that chilling is an autumnal and vernal, rather than an hibernal process. The adaptive significance of chilling as an environmental cue must then be interpreted in view of fall and spring conditions.

The interaction between the chilling and photoperiodic mechanisms in *T. rutilus* may represent an adaptive compromise between selection, due to long-term climate on the one hand and immediate weather conditions on the other. In temperate latitudes during the fall, the consequences of misinterpreting environmental cues are necessarily drastic. Reliance upon cues which maximize average fitness over a number of years will result in the correct phenotype, *i.e.,* diapause (Levins, 1969; Cohen, 1970). Photoperiod is a precise geophysical cue and is used by many insects in late summer and fall for the initiation and maintenance of diapause under otherwise favorable conditions of food and temperature (Lees, 1968; de Wilde, 1962; Danilevskii, 1965; Danilevskii, *et al.,* 1970; Beck, 1968; Tauber and Tauber, 1976a). In the spring, daylength is equally precise but is a less reliable indicator that spring is actually progressing. Spring temperatures, however, are themselves a reflection of individual vernal weather. Cold spring temperatures, below 5° C, would delay the influence of chilling on the photoperiodic mechanism; the larvae would remain deep in diapause, and the critical photoperiod would remain high. The larvae would then be more conservative in their response to subsequent photoperiods, and longer days would be required to elicit a given rate of development than if milder conditions, above 5° but still below 15° C, had prevailed. Consequently, a late winter or cold spring would result in delayed development. An interactive chilling-photoperiod mechanism thus combines the temperature-compensated precision of photoperiodism with a complimentary mechanism that tracks weather during individual springs. Variations on this theme may result in either a combination of events necessary to terminate diapause for all individuals in the population (homeostasis) (Stross, 1971; Adkisson and Roach, 1971; Wellso and Adkisson, 1966; Norris, 1965; Ryan, 1975; Kamm, 1972; Tauber and Tauber, 1976b) or a variety of different events necessary to terminate diapause in different individuals (polymorphism) (Bradshaw, 1973; Waldbauer and Sternberg, 1973; Morris and Fulton, 1970).

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SUMMARY

1. Unchilled, diapausing larvae of *Toxorhynchites rutilus* rely on photoperiod for the maintenance of diapause. The photoperiodic clock is temperature-compensated between 16.5° and 25° C, maintaining both a similar set-joint and inherent accuracy over this range. The rates of development among larvae terminating diapause are dependent upon both temperature and photoperiod.

2. Chilling of dormant *Toxorhynchites rutilus* can promote response to progressively shorter daylengths, thus decreasing the critical photoperiod. Chilling can also accelerate response to long days, thereby decreasing the depth of diapause and, after prolonged exposure, can eventually terminate diapause directly, leaving subsequent morphogenesis independent of photoperiod.

3. The optimal temperature for these effects of chilling is above 4° C, below 16.5° C, and may lie around 7° C.

4. Temperatures between 5° and 15° C are vernal and autumnal rather than hibernal. The interaction between chilling and photoperiod may then represent an adaptive compromise between selection due to long-term climatic trends and the vagaries of spring weather.

LITERATURE CITED

Adkisson, P. L., and S. H. Roach, 1971. A mechanism for seasonal discrimination in the photoperiodic induction of pupal diapause in the bollworm, *Heliothis zea* (Boddie). Pages 272–280 in M. Menaker, Ed., *Biochronometry*. National Academy of Sciences, Washington, D. C.

Andrewartha, H. G., 1952. Diapause in relation to the ecology of insects. *Biol. Rev.*, **27**: 50–107.

Beck, S. D., 1968. *Insect photoperiodism*. Academic Press, New York, 288 pp.

Bradshaw, W. E., 1973. Homeostasis and polymorphism in vernal development of *Chaoborus americanus*. *Ecology*, **54**: 1247–1259.

Bradshaw, W. E., 1974. Photoperiodic control of development in *Chaoborus americanus* with special reference to photoperiodic action spectra. *Biol. Bull.*, **146**: 11–19.

Bradshaw, W. E., and C. M. Holzapfel, 1975. Biology of tree-hole mosquitoes: photoperiodic control of development in northern *Toxorhynchites rutilus* (Coq). *Can. J. Zool.*, **53**: 889–893.

Cohen, D., 1970. A theoretical model for the optimal timing of diapause. *Am. Nat.*, **104**: 389–400.

Danilevskii, A. S., 1965. *Photoperiodism and seasonal development of insects*. Oliver and Boyd, Ltd., London, 282 pp.

Danilevskii, A. S., N. I. Goryshin, and V. P. Tyshchenko, 1970. Biological rhythms in terrestrial arthropods. *Ann. Rev. Entomol.*, **15**: 201–244.

Holzapfel, C. M., and W. E. Bradshaw, 1976. Rearing of *Toxorhynchites rutilus septentrionalis* (Diptera: Culicidae) from Florida and Pennsylvania with notes on their prediapause and pupal development. *Ann. Entomol. Soc. Am.*, **69**: 1062–1064.

Kamm, J. A., 1972. Photoperiodic regulation of growth in an insect: response to progressive changes in day length. *J. Insect Physiol.*, **18**: 1745–1749.

Lees, A. D., 1955. *The physiology of diapause in arthropods*. Cambridge University Press, London, 151 pp.

LEES, A. D., 1968. Photoperiodism in insects. Pages 47–137 in A. C. Giese, Ed., *Photophysiology, Vol. 4,* Academic Press, New York.

LEVINS, R., 1969. Dormancy as an adaptive strategy. *Symp. Soc. Exp. Biol.,* **23**: 1–10.

MCCRARY, A. B., 1965. The effect of photoperiod, temperature, and food on diapausing and developing larvae of *Toxorhynchites rutilus* (Coq.). *M. S. Thesis, University of North Carolina.* Chapel Hill, North Carolina, 49 pp.

MORRIS, R. F., AND W. C. FULTON, 1970. Heritability of diapause intensity in *Hyphantria cunea* and correlated fitness responses. *Can. Entomol.,* **102**: 927–938.

NORRIS, M. J., 1965. The influence of constant and changing photoperiods on imaginal diapause in the red locust (*Nomadacris septemfasciata* Serv.). *J. Insect Physiol.,* **11**: 1105–1119.

OHTAKI, T., R. D. MILKMAN, AND C. M. WILLIAMS, 1968. Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina. Biol. Bull.,* **135**: 322–334.

RYAN, R. B., 1975. Photoperiod effects on development of the larch caseborer, *Coleophora laricella* (Lepidoptera: Coleophoridae). *Can. Entomol.,* **107**: 1305–1310.

SOKAL, R., AND J. ROHLF, 1969. *Biometry.* W. H. Freeman, San Francisco, 776 pp.

STROSS, R. G., 1971. Photoperiod control of diapause in *Daphnia.* IV. Light and CO_2-sensitive phases within the cycle of activation. *Biol. Bull.,* **140**: 137–155.

TAUBER, M. J., AND C. A. TAUBER, 1975. Natural daylengths regulate insect seasonality by two mechanisms. *Nature,* **258**: 711–712.

TAUBER, M. J., AND C. A. TAUBER, 1976a. Insect seasonality: diapause maintenance, termination, and post-diapause development. *Ann. Rev. Entomol.,* **21**: 81–107.

TAUBER, M. J., AND C. A. TAUBER, 1976b. Environmental control of univoltinism and its evolution in an insect species. *Can. J. Zool.,* **54**: 260–265.

TRIMBLE, R. M., AND P. S. CORBET, 1975. Laboratory colonization of *Toxorhynchites rutilus septentrionalis* (Diptera: Culicidae). *Ann. Entomol. Soc. Am.,* **68**: 217–219.

WALDBAUER, G. P., AND J. G. STERNBERG, 1973. Polymorphic termination of diapause by Cecropia: genetic and geographical aspects. *Biol. Bull.,* **145**: 627–641.

WELLSO, S. G., AND P. L. ADKISSON, 1966. A long-day short-day effect in the photoperiodic control of the pupal diapause of the bollworm, *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). *J. Insect Physiol.,* **12**: 1455–1465.

WILDE, J. DE, 1962. Photoperiodism in insects and mites. *Ann. Rev. Entomol.,* **7**: 1–26.

WILLIAMS, C. M., 1956. Physiology of insect diapause X. An endocrine mechanism for the influence of temperature on the diapausing pupa of the Cecropia silkworm. *Biol. Bull.,* **110**: 201–218.

WILLIAMS, C. M., AND P. L. ADKISSON, 1964. Physiology of insect diapause XIV. An endocrine mechanism for the photoperiodic control of pupal diapause in the oak silkworm, *Antheraea pernyi. Biol. Bull.,* **127**: 511–525.

Reference: *Biol. Bull.*, **152**: 159–168. (April, 1977)

THE FORMATION AND TAXONOMIC STATUS OF THE MICROBASIC Q-MASTIGOPHORE NEMATOCYST OF SEA ANEMONES

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Some 27 different types of nematocysts have been described to date from members of the Phylum Cnidaria (Mariscal, 1974c). One of the commonest types, the microbasic mastigophore nematocyst, was described by Weill (1934) as having a well defined shaft (enlarged basal portion of thread) less than three times the capsule length. Carlgren (1940) later subdivided the microbasic mastigophores into two distinct types, the microbasic b- and microbasic p-mastigophores, based on the structure of the shaft. The shaft of a discharged microbasic b-mastigophore tapers gradually into the thread, while that of a discharged microbasic p-mastigophore abruptly narrows down to form the thread (*e.g.,* see Fig. 4–14 of Mariscal, 1974c). The lower portion of the shaft of an undischarged microbasic p-mastigophore has a prominent inverted V-shaped notch, while the microbasic b-mastigophore lacks this feature.

Cutress (1955) has described a third type from sea anemone acontia, the microbasic q-mastigophore, characterized by the possession of a harpoon-like dart which is propelled out of the capsule during discharge. Iwanzoff (1896) was apparently the first to notice this phenomenon, and Weill (1934) has also described it in association with two different nematocysts: the microbasic mastigophore and microbasic amastigophore.

The amastigophore is similar to the microbasic mastigophore, but as originally described, lacked a terminal thread beyond the shaft (Weill, 1934). However, as Cutress (1955) has pointed out, a very short fragment of thread may be present, either on the discharged shaft or left behind attached to the inside of the discharged capsule wall.

Iwanzoff (1896), Weill (1934), Hand (1961) and Schmidt (1969) suggest that the dart represents spines from the shaft which have somehow fused together, or otherwise failed to separate, during discharge. These authors do not consider the presence of a dart to be sufficient grounds for the erection of a new category of nematocyst. Cutress (1955), on the other hand, has argued that the dart is an unattached, discrete structure which is consistently found in the capsules of certain nematocysts and characterizes an entirely new type of nematocyst. In order to resolve this controversy and because darts represent one of the most curious features yet described in association with nematocysts, dart-forming nematocysts were examined in the present study using both phase contrast and scanning electron microscopy. Specific problems addressed include the relative proportion of darts formed by tentacle nematocysts as opposed to acontial and column nematocysts, the type(s) of nematocysts capable of forming darts, the number of darts formed by nematocysts within a particular category, the method of formation of darts and their possible function in the lives of the organisms involved. Based on the information obtained from a variety of sources in the present study, it will be seen that the microbasic q-mastigophore should not be considered as a valid taxonomic entity.

Materials and Methods

The sea anemone material examined in the present study included the tentacles and acontia of *Aiptasia pallida* (Verrill, 1864), the tentacles and acontia of *Bartholomea annulata* (Le Sueur, 1817) and the tentacles and column vesicles of *Bunodeopsis antilliensis* Duerden, 1897. *Aiptasia* was found on floating docks and *Bunodeopsis* on floating *Sargassum* in the vicinity of Alligator Harbor on the north Florida Gulf Coast. *Bartholomea* was collected from coral heads off Key Largo, Florida.

Fixation of tissues for scanning electron microscopy (SEM) was in Parducz's (1967) Fixative. The subsequent preparation for Freon critical point drying followed the procedures of Mariscal (1974a, b). The dried tissue was coated with gold-palladium and then examined in the Cambridge Stereoscan S4–10 scanning electron microscope.

Positive and negative (anoptral contrast) phase contrast microscopy was done on a Reichert Zetopan microscope with a Nikon AFM photomicrographic attachment.

Counts of nematocysts at 400 × magnification were made from three fields of view of two different preparations, each for a total of six counts per nematocyst type observed. Nematocyst terminology follows that given in Mariscal (1974c).

Results

New nematocyst types: microbasic amastigophore, types A and B; microbasic p-mastigophore, types A and B

As Weill (1934) points out, two different types of nematocysts may be involved in dart formation: the microbasic mastigophore (Fig. 1) and the microbasic amastigophore (Fig. 2). Of the former, only the microbasic p-mastigophore is thought to be capable of forming darts, and because of this, Cutress (1955) has erected a new category which he calls the microbasic q-mastigophore.

Both the microbasic p-mastigophore and microbasic amastigophore can be subdivided into two additional types, A and B, based on the size, shape, arrangement and relative abundance of spines on the everted shaft. Although these differences are apparent in phase contrast microscope observations (Fig. 2), SEM observations were required to determine their exact nature.

The microbasic amastigophore type A is characterized by a widely spaced and well-differentiated helical coiling of spines whose length is equal to, or less than, the diameter of the shaft (Fig. 3). The tips of these spines in our preparations are truncated rather than pointed, as they are in many nematocysts.

The shaft of the microbasic amastigophore type B, on the other hand, has many flat, blade-like spines which are as long or longer than the shaft diameter and which have pointed tips (Fig. 3). In addition, either due to spine length or number, their arrangement on the shaft is not obvious, although it is assumed to be a triple helix based on observations of other species (Mariscal, 1974c). Because of the size and shape of its spines, the discharged shaft of the microbasic amastigophore type B tends to have a bottle-brush appearance (Fig. 4).

The microbasic p-mastigophore may also be subdivided into types A and B, using the same criteria as in the case of the microbasic amastigophores. In both cases, only the type B nematocysts (possessing the long, blade-like spines) are capable of forming darts (Table I).

Relative frequency of dart formation from different regions of the anemone

Both the microbasic amastigophore type B and microbasic p-mastigophore type B nematocysts are capable of forming darts. However, depending on the location in the anemone of the type B nematocyst, there is a striking difference in the relative number of darts formed. Dart formation by either type of nematocyst is rare in the tentacles, but relatively common by the same nematocyst types located in the lower column vesicles or acontia as shown by Tables I and II. For example, only 1.1% or less of the type B amastigophores or mastigophores from the tentacles formed darts, while 27.4% and 28.6% of the mastigophores from the acontia formed darts (Table IIA). Regardless of whether the same nematocyst types (*e.g.,* Table IIA, *B. annulata*) or a different nematocyst type (*e.g.,* Table IIA, *A. pallida*) was involved, the nontentacle nematocysts were the only ones which commonly formed darts (*cf.,* Table I).

Mechanism of dart formation

Examination of acontia or column vesicles with the phase contrast microscope revealed darts of varying lengths lying next to microbasic amastigophores and microbasic p-mastigophores whose shafts were partially devoid of spines (Figs. 5, 6, 7). Occasionally the dart could be found poised on the tip of the everted shaft, reminiscent of the detachable head of a spear gun or harpoon (Fig. 8). In some cases, the dart could be observed at the tip of a partially everted shaft (Fig. 9). In such cases, it appeared that eversion of the shaft had been halted part way through discharge, perhaps due to the dart acting as a plug or somehow interfering with the eversion process. The conclusion from these light microscopical observations was that the dart appeared to be formed by a fusion or adhesion of the shaft spines during discharge. This hypothesis was confirmed by the use of scanning electron microscopy.

SEM observations of the tentacles of *Bartholomea annulata* containing microbasic p-mastigophore nematocysts halted part way through discharge clearly showed that the spines had been stripped off the everted shaft for varying distances (Fig. 10). The length of each of the darts in such cases was generally proportional to the length of the denuded portion of the shaft: the shafts bearing short darts had the spines missing for only a short portion of their length and *vice versa* (Figs. 10, 11). A closer examination of the tip of such a shaft clearly shows the contribution of the shaft spines to the formation of the dart, as well as suggesting that failure of some of the spines to sever their connection with the previously everted portion of the shaft may have been responsible for the incomplete eversion of this nematocyst (Fig. 12).

Interestingly enough, it is even possible to determine the point of origin on the shaft of some of the individual spines which form the dart. Note in Figure 13 that some spines incorporated into the dart are intact, having broken free

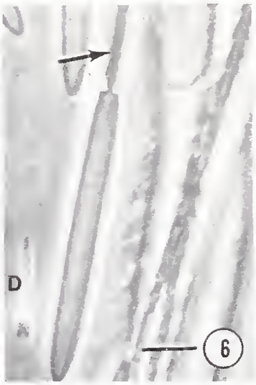
1

B
A
2

A
B
3

4

D
5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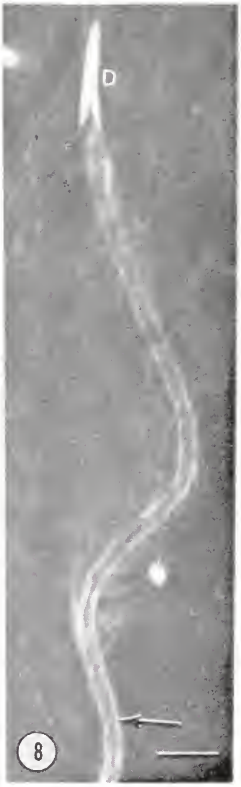
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TABLE I

Tentacle, column and acontial nematocysts of the sea anemones Aiptasia pallida, Bartholomea annulata *and* Bunodeopsis antilliensis *showing the relative frequency of dart formation.*

Species of anemone and nematocyst type	Location in anemone	Relative frequency of dart formation
Aiptasia pallida		
MA, type B	Tentacles	rare
Mp-M, type B	Acontia	common
Bartholomea annulata		
Mp-M, type B	Tentacles	none
Mp-M, type B	Acontia	common
Bunodeopsis antilliensis		
MA, type A	Tentacles	none
MA, type B	Tentacles	rare
MA, type A	Lower column vesicles	none
MA, type B	Lower column vesicles	common

Key: MA = microbasic amastigophore; Mp-M = microbasic p-mastigophore.

at the point where the flanged base of the spine joins the shaft surface (*i.e.,* those spines making up the basal quarter of the dart length). Further along towards the tip of the same dart, it will be seen that the spines became broken during incorporation into the dart. Their stubby basal portions can still be seen adhering to the everted shaft (Figs. 11, 13).

As with length, the shape of the dart may also be variable. In some cases, the dart tapers directly into a sharp point (*e.g.,* Figs. 6, 13), while in other cases it has a slightly enlarged, spear-like tip (Figs. 5, 14).

Possible functions of the dart

In order to test the idea that darts could be propelled with sufficient force to penetrate another organism, free-swimming *Artemia* nauplii were allowed to con-

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FIGURE 1. Phase contrast photomicrograph of microbasic p-mastigophore, type B, from acontium of *Aiptasia pallida.* Scale bar is 10 μm.

FIGURE 2. Phase contrast photomicrograph of microbasic amastigophores, types A and B, from column vesicle of *Bunodeopsis antilliensis.* Scale bar is 5 μm.

FIGURE 3. Scanning electron micrograph (SEM) of microbasic amastigophores, types A and B, from column vesicle of *Bunodeopsis antilliensis.* Scale bar is 1 μm.

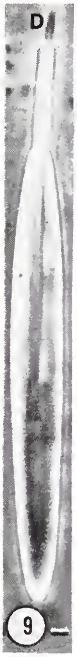
FIGURE 4. SEM of microbasic amastigophores, type B, on tentacle of *Aiptasia pallida.* Scale bar is 5 μm.

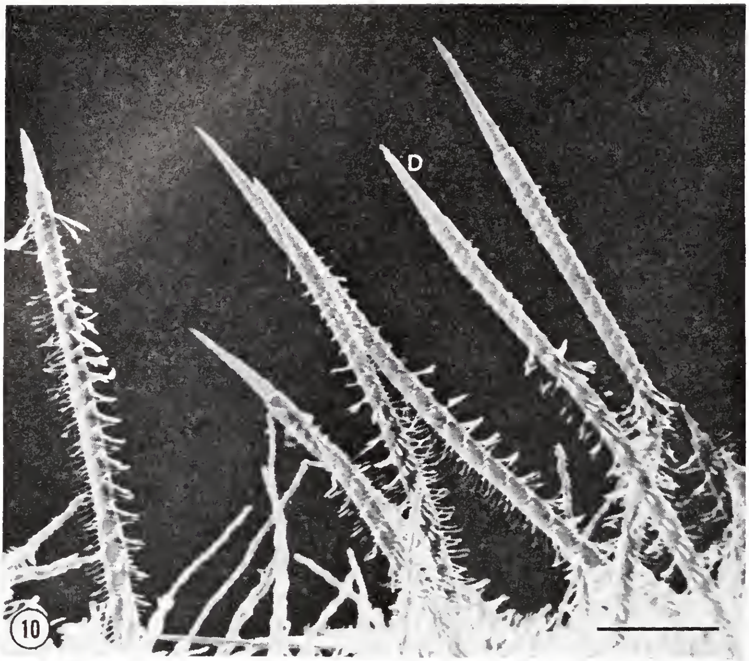
FIGURE 5. Phase contrast photomicrograph of detached dart (D) lying next to shaft denuded of spines (arrow) of microbasic p-mastigophore, type B, from acontium of *Aiptasia pallida.* Scale bar is 10 μm.

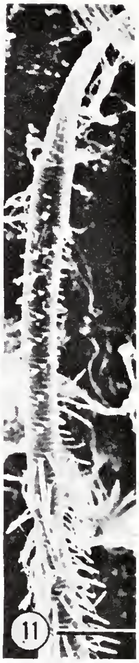
FIGURE 6. Phase contrast photomicrograph of detached dart (D) lying next to capsule of microbasic p-mastigophore, type B, with shaft denuded of spines (arrow) from acontium of *Aiptasia pallida.* Scale bar is 5 μm.

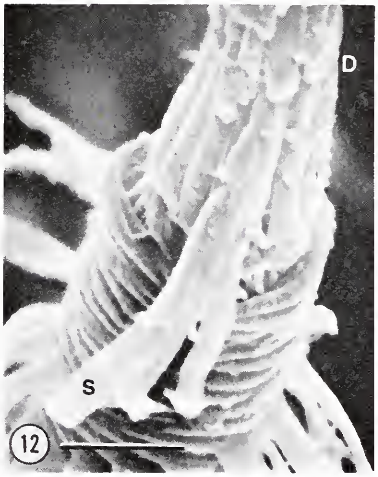
FIGURE 7. Phase contrast photomicrograph of microbasic amastigophore, type B, from column vesicle of *Bunodeopsis antilliensis.* Note short thread (arrow) left attached to inner wall of capsule and the basal portion of shaft lacking spines below dart (D). Scale bar is 5 μm.

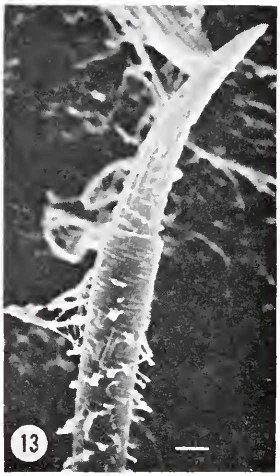
FIGURE 8. Negative phase contrast photomicrograph of everted shaft of microbasic amastigophore, type B, from column vesicle of *Bunodeopsis antilliensis.* Note basal portion of shaft lacking spines (arrow) and dart (D) poised on tip of shaft. Scale bar is 5 μm.











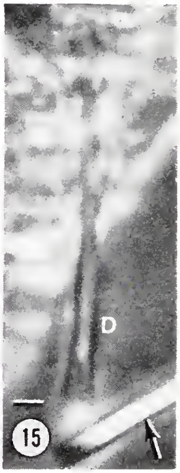


FIGURE 9. Phase contrast photomicrograph of microbasic amastigophore, type B, from tentacle of *Aiptasia pallida* with dart (D) at tip of partially everted shaft. Note that everted portion of shaft lacks spines. Scale bar is 1 μm.

FIGURE 10. SEM of microbasic p-mastigophores, type B, with darts (D) at tips of partially everted shafts on tentacle of *Bartholomea annulata*. Note everted shafts with missing or broken spines. Scale bar is 10 μm.

FIGURE 11. SEM of distal portion of microbasic p-mastigophore, type B, from tentacle of *Bartholomea annulata* showing dart and shaft with numerous missing or broken spines. Scale bar is 4 μm.

FIGURE 12. SEM of partially everted shaft of microbasic p-mastigophore, type B, from tentacle of *Bartholomea annulata* showing incorporation of shaft spines (S) into formation of dart (D). Scale bar is 1 μm.

TABLE II

Nematocysts from different species of sea anemones showing the actual percentage of dart formation under different conditions. Each type of nematocyst was counted at a magnification of 400× using three different fields of view from two preparations each for a total of six counts.

Species of anemone and nematocyst type	Location in anemone	Total number of counts at 400×	Total number of nematocysts	Total number of darts	Per cent darts observed
A. Nematocysts from excised tentacles and acontia					
Aiptasia pallida					
MA, type B	Tentacles	6	175	2	1.1
Mp-M, type B	Acontia	6	190	52	27.4
Bartholomea annulata					
Mp-M, type B	Tentacles	6	95	0	0
Mp-M, type B	Acontia	6	227	65	28.6
B. Nematocysts from *in situ* tentacles and acontia discharged on glass coverslips coated with *Artemia* nauplius extract.					
Bartholomea annulata					
Mp-M, type B	Tentacles	6	94	0	0
Mp-M, type B	Acontia	6	222	54	24.3

Key: MA = microbasic amastigophore; Mp-M = microbasic p-mastigophore.

tact the extruded acontia of *A. pallida* and their exoskeletons examined microscopically to determine if any darts might be present. It was determined that darts could penetrate the exoskeleton of a crustacean (Fig. 15).

It is also possible that some partially discharged darts projecting out at right angles from the tentacle surface could be effective in deterring a potential predator, especially if contact caused these nematocysts to complete eversion and discharge their contained darts (*e.g.,* see Fig. 10).

One might expect that if dart formation had a defensive function, food stimuli might not affect the relative numbers of darts formed. Therefore, the effect of food stimuli on dart formation was also tested in a living *B. annulata* anemone. Food stimulus-coated coverslips (with *Artemia* extract) presented to the intact tentacles and acontia of this anemone did not alter the relative proportion of darts formed by tentacle and acontial nematocysts; similar to the results shown in Tables I and IIA, no darts were formed by the tentacle microbasic p-mastigophores, while 24.3% of this nematocyst type in the acontia did form darts (Table IIB).

FIGURE 13. SEM of tip of partially everted shaft and dart of microbasic p-mastigophore, type B, from tentacle of *Bartholomea annulata*. Note whorls of intact spines forming basal portion of dart. Scale bar is 1 μm.

FIGURE 14. SEM of dart at tip of partially everted microbasic p-mastigophore, type B, on tentacle of *Bartholomea annulata*. Note flanged bases of intact spines at base of dart and enlarged tip of dart. Scale bar is 1 μm.

FIGURE 15. Phase contrast photomicrograph of dart (D) from microbasic p-mastigophore, type B, from acontium of *Aiptasia pallida* embedded in exoskeleton of *Artemia* nauplius larva at left. Note second dart (arrow) lying free in medium next to *Artemia*. Scale bar is 1 μm.

Discussion

As Cutress (1955) points out, and we have been able to verify, many amastigophores do in fact have a very short thread, all or a portion of which may be left behind attached to the inside of the capsule wall following discharge. Thus, at least some amastigophores could be considered to be microbasic mastigophores with very short threads. This has prompted several workers (Cutress, 1955; Schmidt, 1969, 1972, 1974) to propose the elimination of the category of amastigophore entirely. Based on our studies, we can generally sympathize with this point of view. For example, elimination of the amastigophore might make it easier to explain why both the amastigophore and microbasic p-mastigophore can form darts and why each have two sub-types, A and B.

On the other hand, we believe it would be premature at this point to eliminate the amastigophore completely until more nematocysts of this and other types (especially the microbasic p-mastigophores) from additional species can be examined in detail with the electron microscope. In addition, the tentative retention of the amastigophore can perhaps be justified on functional grounds, in that regardless of what it is called, it is at least apparently functioning as an amastigophore since its short thread may often be left behind in the capsule or broken during discharge.

Based on our observations, only the type B amastigophores and microbasic p-mastigophores are capable of forming darts. Their relatively greater density and tighter packing probably allow the longer and more delicate spines of this nematocyst to adhere together and to be stripped from the shaft to form the dart during eversion.

The formation of darts by the type B nematocysts from either the acontia or column vesicles is of interest because of the possible adaptive significance to the species involved. Acontia have generally been considered to have a defensive function because they are often extruded through the body wall when an anemone is disturbed and because they bear large numbers of penetrating nematocysts (Hyman, 1940; McLean and Mariscal, 1973). The fact that darts are commonly formed by nematocysts only from the acontia or column vesicles suggests that they may play some role in the life of the anemones involved, perhaps in defense from benthic predators such as nudibranchs. The latter often contact the column in the vicinity of the vesicles or cinclides (through which the acontia are extruded) during the initial stages of predation (Waters, 1973; Conklin and Mariscal, 1977). Waters (1973), for example, reports that first contact of the predatory nudibranch *Aeolidia* with the acontiate anemone *Metridium* initiated contraction of the anemone and caused its acontia to move toward the nudibranch. Further contact by the nudibranch caused the anemone to contract more strongly and to extrude its acontia through the body wall in the region of contact. Contact of an acontium with the nudibranch caused the latter to contract strongly, especially at the point of contact, and to move away from the acontium while secreting copious amounts of mucus.

Because dart formation was, on the average, some 27 times more frequent in acontial nematocysts than in tentacle nematocysts and because darts can penetrate other organisms, it is suggested that the formation of darts by an anemone may confer some selective advantage to the species. For example, a penetrating nematocyst which anchored prey organisms to an anemone's tentacles would obviously be of importance in preventing prey from escaping before ingestion. However, one would not expect selection to favor the use of the same type of nematocyst against an organism which preyed on anemones. In this case, it would be to the advantage of the anemone simply to deter or drive away a potential predator, perhaps by the use of a detachable spear head or arrow which was *not* anchored to the anemone's tissues.

It is concluded that although the microbasic q-mastigophore does not appear to be a distinct morphological type (since the dart forms from the fused spines of other nematocysts during discharge), it may represent a functional type of nematocyst. Should this be the case, and should dart formation confer some selective advantage to a species, then perhaps we are observing the evolution of a new morphological type of nematocyst. Thus in the tentacles, where selection might favor nematocysts which are capable of injecting toxin and aiding in prey capture, there would be little selective pressure to form darts. On the other hand, in the acontia or column vesicles which might first be contacted by a potential benthic predator, we might expect to, and in fact do, find a much greater frequency of dart formation. An experimental study investigating the relative success among aeolid nudibranchs preying on anemones possessing dart-forming nematocysts and those lacking these cnidae would be of interest in this regard.

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SUMMARY

Detachable darts, supposedly diagnostic of the microbasic q-mastigophore nematocyst, are formed by the adhesion of shaft spines of two different nematocysts: the microbasic amastigophore and microbasic p-mastigophore. Based on the morphology of their shaft spines, each of the nematocysts can be subdivided into two additional types, A and B. Only the type B nematocysts are capable of forming darts.

Dart formation by tentacle nematocysts was rare, but was relatively common among type B acontial or column vesicle nematocysts. Experiments showed that darts were formed by acontial nematocysts, for example, about 27 times more frequently than by tentacle nematocysts, both intra- and interspecifically. However, only a relatively small percentage of type B nematocysts appeared capable of forming darts in the material examined.

Darts were propelled out of the capsule with sufficient force to penetrate crustacean exoskeleton. Because of this and evidence from the experimental studies, it is suggested that dart formation may play a role in the defense of some anemones against benthic predators. Although possibly having functional significance for those anemones possessing the appropriate nematocyst, dart formation caused by the fusion of shaft spines among only a certain percentage of the nematocyst population indicates that the microbasic q-mastigophore is not a valid nematocyst type.

LITERATURE CITED

CARLGREN, O., 1940. A contribution to the knowledge of the structure and distribution of the cnidae in the Anthozoa. *Lunds Univ. Årsskrift, N. F., Avd. 2,* **36**: 1–62.

Conklin, E. J., and R. N. Mariscal, 1977. Feeding behavior, cerata structure and nematocyst storage in the aeolid nudibranch, *Spurilla neapolitana* (Mollusca). *Bull. Mar. Sci.,* **27**, in press.

Cutress, C., 1955. An interpretation of the structure and distribution of cnidae in the Anthozoa. *Syst. Zool.,* **4**: 120–137.

Hand, C., 1961. Present state of nematocyst research: types, structure, and function. Pages 187–202 in H. M. Lenhoff and W. F. Loomis, Eds., *The biology of hydra and of some other coelenterates: 1961.* University of Miami Press, Coral Gables, Florida.

Hyman, L. H., 1940. *The invertebrates: Protozoa through Ctenophora.* Vol. I. McGraw-Hill, New York, 726 pp.

Iwanzoff, N., 1896. Ueber den Bau, die Wirkungsweise und die Entwickelung der Nesselkapseln der Coelenteraten. *Bull. Soc. Nat. Moscou, (N. S.)* **10**: 95–161.

Mariscal, R. N., 1974a. Scanning electron microscopy of the sensory surface of the tentacles of sea anemones and corals. *Z. Zellforsch. Mikrosk. Anat.,* **147**: 149–156.

Mariscal, R. N., 1974b. Scanning electron microscopy of the sensory epithelia and nematocysts of corals and a corallimorpharian sea anemone. *Proc. Second Internat. Coral Reef Symp.,* **1**: 519–532.

Mariscal, R. N., 1974c. Nematocysts. Pages 129–178 in L. Muscatine and H. M. Lenhoff, Eds., *Coelenterate biology: reviews and new perspectives.* Academic Press, New York.

McLean, R. B., and R. N. Mariscal, 1973. Protection of a hermit crab by its symbiotic sea anemone *Calliactis tricolor. Experientia,* **29**: 128–130.

Parducz, B., 1967. Ciliary movement and coordination in ciliates. Pages 91–128 in G. H. Bourne and J. F. Danielli, Eds., *International review of cytology.* Academic Press, New York.

Schmidt, H., 1969. Die Nesselkapseln der Atkinien. *Helgolaender Wiss. Meeresunters.,* **19**: 284–317.

Schmidt, H., 1972. Die Nesselkapseln der Anthozoen und ihre Bedeutung fur die phylogenetische Systematik. *Helgolaender Wiss. Meeresunters.,* **23**: 422–458.

Schmidt, H., 1974. On evolution in the Anthozoa. *Proc. Second Internat. Coral Reef Symp.,* **1**: 533–560.

Waters, V. L., 1973. Food-preference of the nudibranch *Aeolidia papillosa,* and the effect of the defenses of the prey on predation. *Veliger,* **15**: 174–192.

Weill, R., 1934. Contribution a l'étude des cnidaires et de leurs nématocystes. *Trav. Stat. Zool. Wimereux,* **10–11**: 1–701.

Reference: *Biol. Bull.*, **152**: 169–181. (April, 1977)

RADIATION-INDUCED INHIBITION OF ECLOSION IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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Increases in radioresistance in *Drosophila melanogaster* during pupal-adult development were first observed by Mavor (1927) and have since been documented for a number of holometabolous insects (Grosch, 1962 and 1973; Arena, 1971). Mavor (1927, p. 81) concluded that, ". . . the resistance of the pupae to X-rays begins after all of their important organs have been laid down and that their resistance increases with the growth and differentiation of these organs." Preliminary investigations with *Manduca sexta,* X-irradiated at various intervals during pupal-adult development, indicated an increase in radioresistance during this developmental period (Ely and Jungreis, 1976, 1977). Of particular interest in these studies was the observation that X-irradiated animals which failed to eclose were still able to complete metamorphosis.

In the normative scheme of insect development, the sequence of molts and morphological changes characteristic of post-embryonic development is controlled by the interaction of hormones produced by components of the neuroendocrine system (see Wyatt, 1972; Doane, 1973). In holometabolous insects, the last major developmental event in the life cycle is eclosion. Hormonal control of eclosion has been demonstrated in at least three species of silkworms, namely *Hyalophora cecropia, Antheraea polyphemus* and *Antheraea pernyi* (Truman and Riddiford, 1970). Utilizing "loose-brain" animals and brain homogenates, these investigators concluded that the brain was the site of eclosion hormone production. Furthermore, the release of hormone is synchronized with photoperiod regimen and acts on the central nervous system to elicit a species-specific pattern of behavior (pre-eclosion behavior). It is this elicited behavioral response which results in rupture and ecdysis of the pupal cuticle. Pre-eclosion behavior patterns are programmed in the abdominal ganglia rather than the brain (Truman, 1971; Truman and Sokolove, 1972). Eclosion hormone thus serves to trigger the sequence of abdominal movements necessary for emergence. Utilizing pharate adult *A. pernyi* in an assay for eclosion hormone activity, Truman (1973) found high concentrations of the hormone in the brains and corpora cardiaca of *M. sexta* pharate adults on the day of eclosion and provided evidence that the cells in the median neurosecretory cluster of the brain were responsible for eclosion hormone production. Therefore, it is postulated that the radiation-induced inhibition of eclosion observed in *Manduca* results from a disruption of the normal hormonal mechanisms controlling adult emergence.

Studies herein described were undertaken to both analyze in greater detail the effects on adult emergence of X-irradiation of pharate-adults of *M. sexta,* and to assess the neuroendocrine contribution, if any, to observed changes in radiosensitivity exhibited by this species during the pupal-adult transformation.

MATERIALS AND METHODS

Experimental animals

Tobacco hornworms used in these experiments were derived from an inbred colony maintained by Dr. Lynn M. Riddiford, University of Washington, Seattle. Larvae were individually reared in plastic containers on the diet of Yamamoto (1969), modified after Bell and Joachim (1976) and Riddiford (personal communication), under an 18L:6D photoperiod regimen at 23–25° C, according to the methods outlined in Ely and Jungreis (1977).

Irradiation parameters

The X-irradiation source was a General Electric Maxitron 300 X-ray unit operated at 250 kV and 15 mAmp with 0.5 mm aluminum filtration added (4.75 mm Be inherent filtration) to reduce tissue attenuation by 95–100%. Exposures were altered by varying the time of exposure and/or the target-to-object distance. Dosimetry, as measured in air, was determined under experimental conditions utilizing either a 250-r thimble ionization chamber read in a Model 70 Victoreen condenser r-meter, or a Model 575 Victoreen Radocon with medium energy (Model 602) probe.

Animals receiving whole-body or partial-body exposures (in the case of shielding studies) were positioned on a styrofoam platform, 2.5 cm in thickness, during irradiation. Following treatment, all animals were returned to laboratory rearing conditions.

Determination of ED_{50}'s

Animals were divided into three to five treatment groups (25 animals per group) and a control group (25 animals) on each day of the pupal-adult transformation. Treatment groups were given a series of graded whole-body exposures at an exposure rate of approximately 325 r/minute. The range of exposures to which animals were subjected on any given day during development was based on preliminary investigations of radiosensitivity. The fraction of irradiated animals eclosing relative to nonirradiated controls was plotted as a function of exposure and the ED_{50} (X-ray exposure required to prevent 50% of irradiated animals from emerging) determined for each day of pupal-adult development utilizing regression analysis. In this work, eclosion will be defined as those events associated with the emergence of an imago from the confines of the pupal exuvia following completion of pupal-adult development.

Body shielding experiments

Animals were subjected to partial-body irradiation on the tenth day after pupation (day 10) at an ED_{100} level of 48 kr (325 r/minute). A lead plate, 1 cm in thickness, was placed over selected body regions. Exposures in the shielded regions were approximately 1–2% of those measured in the unshielded regions.

Brain transplantation experiments

In a selected group of pharate adults anesthetized with water saturated carbon dioxide (for up to 30 minutes), the brains (supra-esophageal ganglia) were surgically manipulated following procedures slightly modified after Williams (1959). (See Eaton (1974) and Eaton and Dickens (1974) for descriptions of the topography of the brain and central nervous system of *M. sexta.*) A section of cuticle (approximately 2.5 mm by 2.5 mm) was removed from the dorsal regions of the head, and the brain (or brain-corpora cardiaca complexes) either removed or left in place after severing the major nerve trunks leading to the brain. Surgical procedures were carried out with the aid of a dissecting microscope on both non-irradiated animals, and on animals which had just been irradiated with an ED_{100} exposure of 48 kr delivered at 325 r/minute (see Ely and Jungreis, 1977). Prior to each group of operations, dissecting instruments were rinsed in 95% ethanol. Following surgery, a few crystals of an equal part mixture of phenylthiourea and streptomycin sulphate were placed in the wound. Sterile physiologic saline (35 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 200 mM sucrose) was then added to the haemocoel until the level of hemolymph was flush with the surface of the cuticle. The opening was then covered with a plastic window (cut from cellulose acetate cover slips rinsed in 95% ethanol) and sealed in place with hot paraffin wax. Animals were then returned to the insectory. After 24 hours, pharate adults were checked for the presence of trapped air. If air bubbles were visible through the plastic window, the wound was opened and resealed as described above.

Intact brains were implanted into the abdomens of treated and control animals by cutting a small opening in the next to last abdominal segment and inserting the tissue. Procedures for abdominal cuticle excision and wound closure were similar to those described above for head surgery.

Ecdysone administration

Anhydrous 20(R)-hydroxyecdysone (β-ecdysone) (Sigma Chemical Corporation) was diluted with sterile 0.1 M KCl saturated with phenylthiourea to give a solution having a final concentration of 1 microgram ecdysone per microliter.

Animals were individually weighed, anesthetized with carbon dioxide, and injected by means of a 25 microliter Hamilton syringe with β-ecdysone through the mesothoracic tergum on days 1, 4, and 12 of pharate-adult development. The injection site was then sealed with paraffin wax and the animals returned to laboratory rearing conditions. The quantity of β-ecdysone injected was based upon the volume of hemolymph which was assumed to be 40% of body wet weight. This value was used rather than the 50% determined for *H. cecropia* (Jungreis and Wyatt, 1972) or *Antheraea pernyi* (Cherbas and Cherbas, 1970) because of the greater contribution of the pupal cuticle in *M. sexta* to the body weight.

Following administration of ecdysone, animals were irradiated as follows. First, day 1 animals receiving 4 μg of β-ecdysone per gram of wet weight were X-irradiated at ED_0 (see Ely and Jungreis, 1977) with an exposure of 8.23 kr (325 r/minute) at 6, 24, and 48 hours post-injection. One group of animals, injected on day 1 with 4 μg ecdysone per gram of wet weight, received a second ecdysone injection (again, 4 μg/gram wet weight) 24 hours after the first. This group was then subjected to an 8.23 kr X-ray exposure (325 r/minute) 24 hours following the second ecdysone administration. Day 1 animals receiving 4 μg ecdysone/gram wet weight without subsequent irradiation served as a control group. Secondly, day 12 animals were treated in a manner identical to that used for day 1 animals with the exception that the ED_0 X-irradiation exposure was 25 kr (325 r/minute). Thirdly, day 4 animals were treated in a manner similar to day 1 animals except that at 6, 12, 24, 36, and 48 hours post ecdysone injections the animals were irradiated at an ED_0 level. Fourthly, day 4 animals were X-irradiated with an exposure of 8.23 kr (325 r/minute) 24 hours following administration of 2, 4, 6, 12, and 16 micrograms of ecdysone per gram of wet weight.

Eclosion success was determined for each experimental group and expressed as a percentage of eclosion success observed in noninjected animals irradiated at similar time intervals. Day 4 and day 12 data were analyzed using single classification analysis of variance. The Student-Newman-Keuls test was employed for a comparison among means (Sokal and Rohlf, 1969).

RESULTS

Effects of X-irradiation on eclosion

The effect of X-irradiation on eclosion of *M. sexta* during the pupal-adult transformation was investigated by initially determining the ED_{50} for animals on each day of development and by utilizing the ED_{50} values as a relative measure of radiation sensitivity (Fig. 1). An initial period of nearly constant radiosensitivity

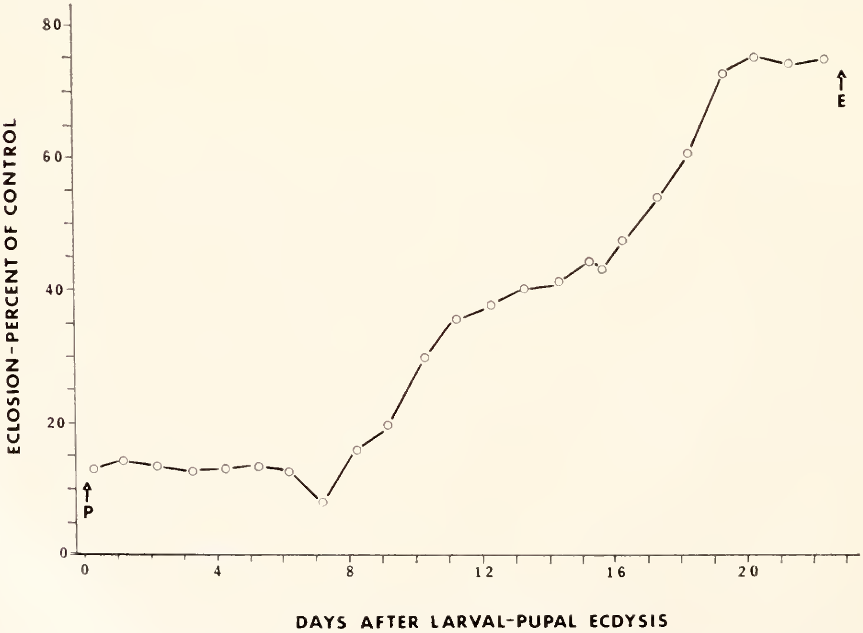

FIGURE 1. ED_{50} values (X-ray exposures required to prevent eclosion in 50% of the irradiated animals) during pupal-adult development of *Manduca sexta*.

was noted between days 0 and 6 (ED_{50}'s ranged from 13.0 kr on days 0 and 6 to 14.4 kr on day 1). This period of constant sensitivity was followed by a brief period of increased radiosensitivity (ED_{50} on day 7 of 8.23 kr) and in turn followed by a pronounced decrease in radiosensitivity through the day of eclosion (day 8: $ED_{50} = 19$ kr; day 22: $ED_{50} = 75$ kr).

The timing of maximum sensitivity on day 7 was determined with greater precision by X-irradiating pharate adults at an exposure level of 8.23 kr (the ED_{50} for day 7) at 6-hour intervals between day 6 and day 8. The increasing radiosensitivity begins 6.25–6.50 days following pupation, reaches a maximum level between 7.25 and 7.50 days, and then returns to a level approximating that seen 6.0–6.25 days following pupation (Fig. 2).

Brain extirpation/transplantation experiments

The role of the brain as a radiosensitive component in animals following the period of maximum radiosensitivity (*i.e.*, day 7) was investigated following brain extirpation and brain transplantation experiments performed on irradiated (with an ED_{100} exposure of 48 kr) and nonirradiated day 10 animals.

Debraining of nonirradiated animals resulted in only 3% eclosion success (Table I). Clearly, the brain is required for successful adult emergence. However, connectives leading from the brain to other regions of the central nervous system must also be intact if eclosion is to occur, since cutting the connectives to the brain of nonirradiated animals resulted in only 45% eclosion success.

Irradiation of day 10 animals with an exposure of 48 kr (ED_{100} exposure) resulted in 0% eclosion success. Implantation of nonirradiated brains into the abdomens of ED_{100} animals did not improve eclosion success, while removal of

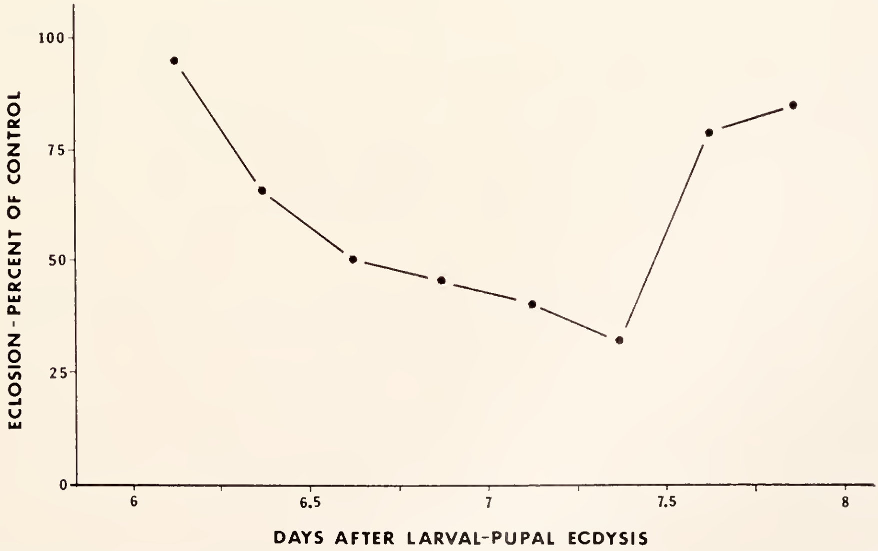

FIGURE 2. Eclosion of *Manduca sexta* X-irradiated during pupal-adult transformation with an exposure of 8.23 kr; 25 animals per exposure group.

TABLE I

Eclosion of Manduca sexta *following surgical manipulation of the brain and/or X-irradiation with an* ED_{100} *exposure of 48 kr on day 10 of pupal-adult development.*

Group number	Treatment	Eclosion	
		Number	Per cent
	Nonirradiated animals		
1	No surgical manipulation	20/20	100
2	Debrained	1/31	3
3	Debrained; nonirradiated brain implanted into head	9/20	45
4	Debrained; ED_{100} brain implanted into head	1/15	7
5	ED_{100} brain implanted into abdomen of animal whose own brain intact	15/15	100
6	Nerve connections to the brain severed	9/20	45
	ED_{100}-irradiated animals		
7	No surgical manipulation	0/20	0
8	Debrained following irradiation; nonirradiated brain implanted into head	1/15	7
9	Nonirradiated brain implanted into abdomen of animal whose own brain was intact	0/20	0

brains from ED_{100} animals following exposure with subsequent implantation of nonirradiated brains resulted in only 7% eclosion success.

This suggests that radiation damage to the brain alone cannot completely account for the 0% eclosion success observed in Group 7 of Table I. However, it is also clear that the brain is "damaged" by irradiation, and that this damage reduces the chance for successful eclosion, as evidenced by comparing the results obtained from Group 3 and Group 4 in Table I. Adding nonirradiated brains to debrained, nonirradiated animals results in a 45% eclosion success. Adding brains irradiated at the ED_{100} level to debrained, nonirradiated animals, however, results in only 7% eclosion success. The 100% eclosion success observed in Group 5, Table I, suggests that the decrease in emergence noted in irradiated animals cannot be attributed to an "active inhibition" of eclosion by the irradiated brain itself.

To determine whether radiation-induced brain damage was accompanied by a decrease in eclosion hormone activity in brains and corpora cardiaca of X-irradiated *M. sexta* pharate adults, animals irradiated with an ED_{100} exposure of 48 kr on day 10, along with nonirradiated animals, were sent to Dr. James W. Truman, University of Washington, Seattle, for biological assay of eclosion hormone (Truman, 1973). In the X-irradiated group of animals, 46% of the brains and 31% of the corpora cardiaca assayed positive for eclosion hormone. This is in marked contrast to the nonirradiated group in which 75% of the brains and 64% of the corpora cardiaca gave positive responses indicating the presence of eclosion hormone activity.

Effects of body shielding on eclosion success

Partial body irradiations of day 10 animals, utilizing an ED_{100} exposure of 48 kr, were performed to identify sensitive and tolerant body regions. Eclosion success following selective shielding is summarized in Table II.

Whole body exposure at 48 kr resulted in 0% eclosion success. Shielding of the abdomen alone resulted in 10% emergence, while 100% of the animals whose head and thorax were simultaneously shielded during irradiation successfully eclosed.

Comparison of Groups 2 and 3 with Group 5 of Table II indicates that the improved eclosion success observed in these former groups can be attributed to shielding of the thorax (Group 2) or shielding of the head (Group 3). Since shielding of the head alone (Group 4) results in 30% eclosion success, it appears that, of the three body regions shielded, the thorax is the most radiosensitive, with the abdomen being least sensitive (Group 1), and the head region being of intermediate radiosensitivity.

Effects of β-ecdysone on eclosion

The possible association between the maximum period of radiosensitivity observed in day 7 animals and an elevated level of endogenous ecdysone (see Kaplanis, Thompson, Yamamoto, Robbins, and Louloudes, 1966) was investigated by injecting day 1, day 4, and day 12 animals with 4 micrograms of β-ecdysone per gram of wet weight and irradiating at specific intervals post-injection with the maximal level of radiation that failed to interfere with eclosion (ED_0 exposure). β-Ecdysone has previously been shown to be the active form of the molting hormone controlling post-embryonic growth and development in tobacco hornworms (Kaplanis *et al.,* 1966; King, Bollenbacher, Borst, Vedeckis, O'Connor, Ittycheria, and Gilbert, 1974).

Administration of ecdysone to days 1, 4, and 12 animals, without subsequent irradiation, resulted in mean eclosion successes of 87%, 87%, and 93%, respectively (Table III). Significant increases in radiosensitivity ($P < 0.05$) were noted in day 4 animals 12 and 24 hours post-injection; and in day 12 animals 6 hours post-injection. Irradiation of day 1 animals following a double-injection routine also increases radiosensitivity as indicated by 53% eclosion success.

TABLE II

Eclosion following partial-body X-irradiation of day 10 Manduca sexta *with an exposure of 48 kr.*

Group number	Body region shielded	Eclosion	
		Number	Percentage of control
1	Head and thorax	20/20	100
2	Thorax and abdomen	18/20	90
3	Head and abdomen	14/20	70
4	Head only	6/20	30
5	Abdomen only	2/20	10
6	No shielding	0/20	0

TABLE III

Eclosion of day 1, day 4, and day 12 Manduca sexta *pharate adults X-irradiated with ED_0 exposures of 8.23 kr, 8.23 kr, and 25 kr, respectively, following injection of 4 micrograms of β-ecdysone per gram wet weight. Results are expressed as percentages of noninjected, irradiated controls.*

Treatments	Eclosion				
	Day 1 animals	Day 4 animals		Day 12 animals	
		Series 1	Series 2	Series 1	Series 2
Injected, nonirradiated	87 (13/15)	80 (12/15)	93 (14/15)	93 (14/15)	93 (14/15)
Irradiated at the following intervals post-injection:					
6 hours	87 (13/15)	60 (12/20)	87 (13/15)	40 (6/15)	53 (8/15)
12 hours	—	30 (6/20)	40 (6/15)	–	—
24 hours	73 (11/15)	47 (7/15)	55 (11/20)	87 (13/15)	100 (15/15)
36 hours	—	72 (13/18)	73 (11/15)	67 (10/15)	40 (6/15)
48 hours	93 (14/15)	100 (15/15)	93 (14/15)	60 (9/15)	47 (7/15)
Irradiated 24 hours following double injection	53 (8/15)	67 (10/15)	60 (9/15)	60 (9/15)	73 (11/15)

The degree of radiosensitivity observed in day 4 animls irradiated 24 hours post-injection is clearly dependent upon the quantity of ecdysone administered (Fig. 3). All animals irradiated 24 hours following injection of 12 and 16 micrograms of

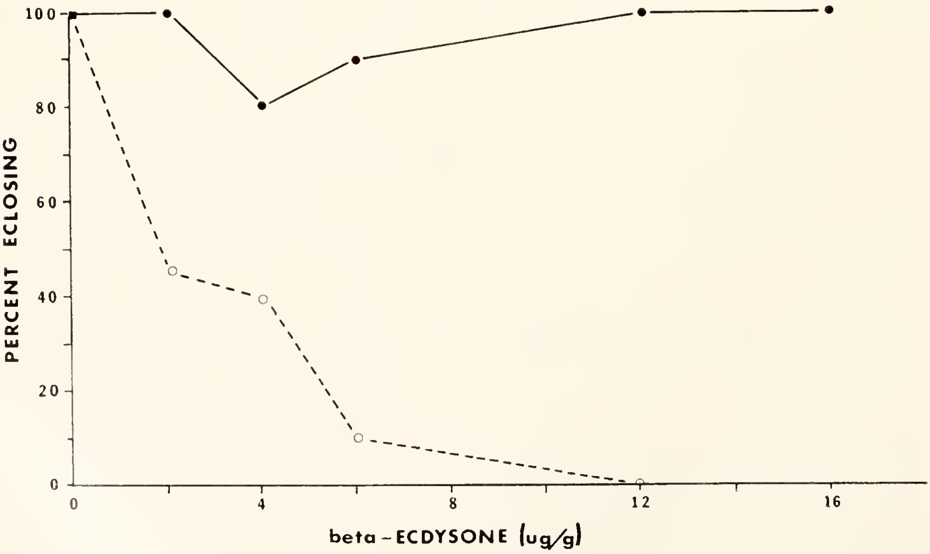

FIGURE 3. Eclosion of *Manduca sexta* following injections of β-ecdysone on day 4 of pupal-adult transformation: (A) nonirradiated; (B) X-irradiated with an exposure of 8.23 kr 24 hours post-injection; 20 animals per treatment group.

ecdysone per gram of wet weight failed to eclose while those receiving identical ecdysone doses but not irradiated exhibited 100% eclosion success.

DISCUSSION

Evaluating the action of X-irradiation of *Musca domestica* pupae upon eclosion, Rockstein, Dauer and Bhatnager (1965) proposed two possible mechanisms to account for the reduced emergence of irradiated flies; namely, premature termination of pupal-adult development, and interference with the actual process of eclosion possibly brought about by damage to the muscle systems involved in the rupture and shedding of the pupal cuticle. These investigators considered the first mechanism to be of minor importance over the exposure range utilized for two reasons; namely, that unemerged, irradiated puparia contained fully formed adults, and that partial eclosion was observed in some animals. Preliminary investigations with *M. sexta* indicated that X-irradiation during the pupal-adult transformation normally inhibited eclosion without disrupting the pupal-adult metamorphosis (Ely and Jungreis, 1977).

In *H. cecropia, A. pernyi,* and *A. polyphemus,* specific hormones are involved in eclosion (Truman and Riddiford, 1970). Therefore, it was initially assumed that the pattern of radiosensitivity (as measured by the ED_{50} values) in *M. sexta* X-irradiated during the pupal-adult transformation would reflect changes in the radiation sensitivity of some component or phase in hormone production. Were the control of eclosion hormone synthesis and release in *M. sexta* similar to that of *A. pernyi* (Truman, 1973), then the observed pattern of radiosensitivity might reflect damage of neurosecretory cells responsible for eclosion hormone production, storage and/or release. To date, a quantitative assay for measuring the titer of eclosion hormone in *M. sexta* has not been reported. Therefore, a direct correlation between radiosensitivity and eclosion hormone levels can not be made.

The titers of ecdysone during pharate-pupal and pharate-adult development have been measured in the tobacco hornworm by several investigators (Kaplanis, *et al.*, 1966; Bollenbacher, Vedeckis, Gilbert and O'Connor, 1975). Employing a biological assay system, Kaplanis and co-workers (1966) monitored changes in ecdysone levels during pupal-adult development in hornworms reared at 24 ± 2° C (eclosion on day 21) and noted a single peak with the maximum titer occurring 6–8 days after the larval-pupal ecdysis. Since the period of maximum ecdysone titer coincided with that of maximum radiosensitivity (day 7) (determined *via* ED_{50}'s), the existence of a positive relationship between these variables could be inferred. The observed relationship between ecdysone and eclosion hormones can be explained in at least two ways; namely, that synthesis of β ecdysone is interrupted by radiation exposure, and that the increased presence of ecdysone renders the animal more sensitive to the effects of radiation. The first model is clearly incorrect, since 93% of irradiated day 7 animals which did not eclose were noted to have completed pupal-adult development. The time course of this development was identical to that observed in nonirradiated animals. Observed increases in radiosensitivity in day 1, day 4, and day 12 animals injected with β-ecdysone (Table III) provide support for the second model. However, the temporal pattern of response in these groups may be dependent upon both the competence of the animal to respond to exogenous β-ecdysone and the titer of endogenous

β-ecdysone at the time of injection and subsequent irradiation. For example, whereas increases in radiosensitivity in days 4 and 12 animals occurred 12 hours and 6 hours post-injection, respectively, that in day 1 animals was elicited only after a double injection of ecdysone (Table III). In this regard, Kaplanis and co-workers (1966) found that the titer of ecdysone in day 1 animals is only 60% of that found in day 4 and day 12 animals. Since the observed radiosensitivity induced by exogenous ecdysone appears to be dose-dependent, then the *total* quantity (both exogenous and endogenous) of β-ecdysone present in hemolymph would be substantially lower in the day 1 groups receiving single injections relative to day 4 and day 12 groups.

The mode of action of ecdysone in effecting the increase in radiosensitivity was not investigated directly. One explanation involves the possible role of ecdysone as a messenger responsible for "turning on" eclosion hormone production in the brain. Irradiation of the brain following ecdysone stimulation could interfere with those critical biochemical activities (such as mRNA synthesis) required for eclosion hormone synthesis, storage, and release.

Eclosion hormone in *M. sexta* is thought to be synthesized by the median neurosecretory cells of the brain and stored in the corpora cardiaca prior to its release into hemolymph at the time of eclosion (Truman, 1973). After initially demonstrating that a hormone stored in the brain was responsible for eclosion in the silkmoth, *H. cecropia,* Truman and Riddiford (Truman and Riddiford, 1970, 1974; Truman, 1971) observed that its presence was not an absolute requirement for eclosion, since animals debrained during diapause or on days 0–3 of pharate adult development were still able to eclose. However, the coordinated preeclosion behavioral pattern characteristic of this species was observed in only 12% of such surgically manipulated animals.

Working with hibernally diapausing *M. sexta,* Judy (1972) reported that animals debrained 20 to 50 days after the larval-pupal ecdysis were able to terminate diapause and complete adult development, but not successfully eclose, observations consistent with those recorded in Table I. In apparent contrast to these results, Wilson and Larsen (1974) observed a 70% rate of eclosion success in *M. sexta* pupae, whose brains were removed at selected early, middle, and late pupal stages. Since Judy (1972) and Wilson and Larsen (1974) debrained *M. sexta* at different stages in development, their results are not comparable.

The role of the brain in regulating eclosion in *M. sexta* was determined by carrying out brain extirpation and exchange experiments in conjunction with selective shielding studies on irradiated and nonirradiated animals at day 10 of pupal-adult development. The eclosion success observed in debrained, nonirradiated day 10 animals was only 3%, a value in marked contrast to the 70% observed by Wilson and Larsen (1974). This finding is also at variance with results obtained with debrained *H. cecropia* (Truman, 1971), but is consistent with results obtained by Judy (1972) for *M. sexta.* Furthermore, the 45% eclosion success seen in animals following simple brain exchange and following severence of the major nerve trunks to the brain indicates that the role of the brain in controlling eclosion cannot be explained entirely in terms of hormonal mechanisms. For example, the zero level eclosion success observed in day 10 animals following a 48 kr X-ray exposure is not merely the result of radiation damage to the brain, since implantation of nonirradiated brains into the heads of animals debrained following irradiation or implantation of nonirradiated brains into the abdomens of animals whose own brains remained intact did not significantly improve eclosion success. Had the brain been the sole target of radiation damage, one would have expected an eclosion success of at least 45% in each of these groups. The 7% eclosion success observed in nonirradiated, debrained animals into which ED_{100} brains were implanted clearly demonstrates that radiation damage affecting eclosion does occur to the brain. Were this not the case, a 45% eclosion success would also have been expected in this group.

In an attempt to identify radiosensitive body regions without resorting to surgical manipulation, selected irradiations at the ED_{100} level of day 10 animal body regions were performed (see Table II). Little enhancement in eclosion success was observed in abdomen-shielded animals. This suggests that any radiation damage in this body region, including damage to the abdominal neurocircuitry and musculature involved in eclosion behavior, does not significantly decrease the chances for successful emergence. Simultaneous shielding of the head and thorax, on the other hand, resulted in a 100% eclosion success. Furthermore, these data suggest that the relative contribution of shielding the thorax is greater than the head shielding in decreasing radiation-induced inhibition of eclosion. Again, radiation damage to the head (including the brain), although a contributory factor, cannot fully account for the decrease in eclosion success observed in these experimental groups.

Truman (1973) has shown that the information required for the nonrepetitive sequence of motor acts involved in eclosion is built into components of the central nervous system which reside outside of the brain. The possibility thus exists that the extensive radiation damage to these components could result in an inhibition of eclosion even should a sufficient quantity of eclosion hormone be present. Williams (1969) has examined the contribution of the thoracic and abdominal ganglia to pupal-adult morphogenesis and emergence using denervated silkworm pupae (*H. cecropia* and *A. polyphemus*). Pupal excision of the central nervous system (brain, subesophageal ganglion, thoracic ganglia, and abdominal ganglia) resulted in termination of adult development. Implantation of loose brains from chilled pupae into such denervated animals caused them to initiate and complete adult development but failed to induce eclosion. Inspection of these flaccid moths revealed no new muscle formation but complete morphogenesis of all other internal organs and integumentary structures. These results indicate that muscle differentiation requires some influence derived from the central nervous system in addition to the hormonal influence supplied by the brain.

The radiation-induced inhibition of eclosion observed in *M. sexta* can be attributed to damage of abdominal ganglia and associated musculature, but this seems unlikely when one considers the aforementioned relative radioresistance of this region. Further, approximately 50% of the irradiated animals which failed to complete the emergence sequence were able to shed the abdominal portion of the pupal exuvia. The specific contribution of radiation damage to the thoracic ganglia is currently under investigation. However, the decrease in eclosion hormone activity observed in brains and corpora cardiaca of ED_{100}-irradiated animals, along with the radiosensitivity exhibited by the brain in both shielding experiments and brain transplantation studies, suggest that radiation damage to the hormonal regulation of eclosion is involved.

In conclusion, the pattern of radiosensitivity observed in *M. sexta* during pupal-adult development reflects specific interference in the regulation of eclosion. The observed radiosensitivity is, in part, dependent upon the stage in development and decreases markedly just prior to eclosion. It is proposed that the radiosensitivity observed during early pupal-adult development (days 1–7) is in part dependent upon the endogenous ecdysone titer and may be related to a "switching on" of eclosion hormone synthesis within the brain by rising ecdysone levels. Studies utilizing brain exchange or shielding clearly demonstrate that, although the brain is a radiosensitive region, a second more sensitive component located in the thorax is also present. Lastly, although hormonally controlled, successful eclosion of nonirradiated *M. sexta* appears to require the presence of an intact brain.

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SUMMARY

Control of eclosion in *Manduca sexta* (laboratory reared at 23–25° C on 18L:6D) was investigated by utilizing the ED_{50} (X-ray exposure required to prevent eclosion in 50% of the irradiated animals) throughout pupal-adult development as a measure of radiation sensitivity. An initial period (day 0–6) of nearly constant radiosensitivity (ED_{50} range: 13.0–14.4 kr) was followed by a brief period of increased radiosensitivity between day 6.5–7.75 (ED_{50}: 8.23 kr). Thereafter, a pronounced decrease in radiosensitivity was noted through the day of eclosion (day 8: $ED_{50} = 19$ kr; day 22: $ED_{50} = 75$ kr).

The association between hemolymph ecdysone levels and maximum radiosensitivity observed on day 7 was studied. Animals administered β-ecdysone on days 1, 4, and 12, and irradiated at various times post-injection, exhibited significant increases in radiosensitivity. Thus, radiosensitivity exhibited by *Manduca sexta* on days 0–7 is in part dependent upon the titer of ecdysone in hemolymph.

The role of the brain as a radiosensitive region was investigated in day 10 animals by selectively transplanting ED_{100}-irradiated and nonirradiated brains into ED_{100}-irradiated and nonirradiated animals. The presence of a radiosensitive component in addition to the brain is proposed since the radiation-induced inhibition of eclosion could not be completely explained in terms of brain damage alone.

Selective shielding of day 10 animals X-irradiated at an ED_{100} level demonstrated the absence of radiosensitive regions in the abdomen and their presence in both the head and thorax.

LITERATURE CITED

ARENA, V., 1971. *Ionizing radiation and life.* C. V. Mosby, St. Louis, 543 pp.

BELL, R. A., AND F. G. JOACHIM, 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Am.*, **69**: 365–373.

Bollenbacher, W. E., W. V. Vedeckis, L. I. Gilbert, and J. D. O'Connor, 1975. Ecdysone titers and prothoracic gland activity during the larval-pupal development of *Manduca sexta*. *Dev. Biol.*, **44**: 46–53.

Cherbas, L., and P. Cherbas, 1970. Distribution and metabolism of alpha-ecdysone in pupae of the silkworm *Antheraea polyphemus*. *Biol. Bull.*, **138**: 115–128.

Doane, W. W., 1973. Role of hormones in insect development. Pages 291–497 in S. J. Counce and C. H. Waddington Eds., *Developmental insect systems, Vol. 2*. Academic Press, New York.

Eaton, J. L., 1974. Nervous system of the head and thorax of the adult tobacco hornworm, *Manduca sexta*. *Int. J. Insect Morphol. Embryol.*, **3**: 47–66.

Eaton, J. L., and J. C. Dickens, 1974. Retrocerebral endocrine glands of the adult tobacco hornworm, *Manduca sexta* (L.). *Int. J. Insect Morphol. Embryol.*, **3**: 273–278.

Ely, M. J., and A. M. Jungreis, 1976. Effects of X-irradiation on eclosion in developing tobacco hornworms, *Manduca sexta*. *Physiologist*, **19**: 180.

Ely, M. J., and A. M. Jungreis, 1977. The effects of X-irradiation on egg hatchability and larval survival in the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphyngidae). *J. Insect Physiol.*, **23**: in press.

Grosch, D. S., 1962. Entomological aspects of radiation as related to genetics and physiology. *Annu. Rev. Entomol.*, **7**: 81–106.

Grosch, D. S., 1973. Environmental aspects of radiation. Pages 85–126 in M. Rockstein, Ed., *The physiology of Insecta, 2nd Edition, Vol. II*. Academic Press, New York.

Judy, K. J., 1972. Diapause termination and metamorphosis in brainless tobacco hornworms (Lepidoptera). *Life Sci.*, **11**: 605–611.

Jungreis, A. M., and G. R. Wyatt, 1972. Sugar release and penetration in insect fat body: Relations to regulation of haemolymph trehalose in developing stages of *Hyalophora cecropia*. *Biol. Bull.*, **143**: 367–391.

Kaplanis, J. N., M. J. Thompson, R. T. Yamamoto, W. E. Robbins, and S. J. Louloudes, 1966. Ecdysones from the pupa of the tobacco hornworm, *Manduca sexta* (Johannson). *Steroids*, **8**: 605–623.

King, D. S., W. E. Bollenbacher, D. W. Borst, W. V. Vedeckis, J. D. O'Connor, P. I. Ittycheria, and L. I. Gilbert, 1974. The secretion of alpha-ecdysone by the prothoracic glands of *Manduca sexta in vitro*. *Proc. Natl. Acad. Sci. U.S.A.*, **71**: 793–796.

Mavor, J. W., 1927. A comparison of the susceptibility to X-rays of *Drosophila melanogaster* at various stages of its life-cycle. *J. Exp. Zool.*, **47**: 62–83.

Rockstein, M., M. Dauer, and P. L. Bhatnagar, 1965. Adult emergence of the house fly, *Musca domestica*, from X-irradiated pupae. *Ann. Entomol. Soc. Am.*, **58**: 375–379.

Sokal, R. R., and F. J. Rohlf, 1969. *Biometry*. W. H. Freeman and Co., San Francisco, 776 pp.

Truman, J. W., 1971. Physiology of insect ecdysis. I. The eclosion behaviour of saturniid moths and its hormonal release. *J. Exp. Biol.*, **54**: 805–814.

Truman, J. W., 1973. Physiology of insect ecdysis. II. The assay and occurrence of the eclosion hormone in the Chinese oak silkmoth, *Antheraea pernyi*. *Biol. Bull.*, **144**: 200–211.

Truman, J. W., and L. M. Riddiford, 1970. Neuroendocrine control of ecdysis in silkmoths. *Science*, **167**: 1624–1626.

Truman, J. W., and L. M. Riddiford, 1974. Hormonal mechanisms underlying insect behaviour. *Adv. Insect Physiol.*, **10**: 297–352.

Truman, J. W., and P. G. Sokolove, 1972. Silkmoth eclosion: hormonal triggering of a centrally programmed pattern of behavior. *Science*, **175**: 1491–1493.

Williams, C. M., 1959. The juvenile hormone. I. Endocrine activity of the corpora allata of the adult cecropia silkworm. *Biol. Bull.*, **116**: 323–338.

Williams, C. M., 1969. Nervous and hormonal communication in insect development. *Dev. Biol.*, **3**: 133–150.

Wilson, G. R., and J. R. Larsen, 1974. Debraining and diapause development in *Manduca sexta* pupae. *J. Insect Physiol.*, **20**: 2459–2473.

Wyatt, G. R., 1972. Insect hormones. Pages 385–490 in G. Litwak, Ed., *Biochemical actions of hormones, Vol. II*. Academic Press, New York.

Yamamoto, R. T., 1969. Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *J. Econ. Entomol.*, **62**: 1427–1431.

Reference: *Biol. Bull.*, **152**: 182–198. (April, 1977)

TEMPERATURE RELATIONS OF AERIAL AND AQUATIC RESPIRATION IN SIX LITTORAL SNAILS IN RELATION TO THEIR VERTICAL ZONATION[1]

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All larger intertidal animals are alternately aerial and aquatic in habit. The majority of littoral molluscs (surprisingly including bivalves as well as gastropods) are adapted for respiration both as air-breathers and by aquatic ventilation of ctenidia or other "gill" structures. Temperature relations of aquatic respiration in one of the most common littoral snails, *Littorina littorea* have been extensively investigated (Newell, 1969, 1973; Newell and Pye, 1970a, b, 1971a, b; Pye and Newell, 1973; Newell and Roy, 1973), and the occurrence of some degree of regulation in "standard metabolism" has been established for that species (Newell and Pye, 1970b; Pye and Newell, 1973). Comparative studies on congeneric and other related intertidal snails are less extensive (Sandison, 1966, 1967; Toulmond, 1967a, b). The present report deals with temperature-induced shifts in oxygen uptake rates for the aquatic respiration of six (and the aerial respiration of four) species of littoral and sublittoral snails. Parallel studies (McMahon and Russell-Hunter, 1974, 1977) on responses to low oxygen stress revealed considerable interspecific differences, but these were less related to vertical zonation on seashores than to microenvironmental factors and to the physiological adaptations of each individual species. With interspecific (and even with infraspecific) differences in temperature relations, we are somewhat more directly concerned with degrees of aerial exposure and hence with the principal patterns of their distributional ecology including intertidal vertical zonation.

Using oxygen electrodes, respiration rates in sea water over a range of natural temperatures were determined for *Acmaea testudinalis, Mitrella lunata, Lacuna vincta, Littorina obtusata, L. littorea,* and *L. saxatilis.* Corresponding aerial respiration rates were determined for *Acmaea* and for the three *Littorina* spp. Early results in these investigations were reported in two preliminary abstracts (McMahon and Russell-Hunter, 1973; Russell-Hunter and McMahon, 1974).

MATERIALS AND METHODS

Determinations of oxygen uptake rates were made for six species of marine prosobranch gastropods: three sublittoral species, *Lacuna vincta, Mitrella lunata,* and *Acmaea testudinalis;* and three intertidal species, *Littorina obtusata, Littorina*

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littorea, and *Littorina saxatilis.* Current American usage is adopted in the nomenclature of the three common *Littorina* spp., *L. obtusata, L. littorea,* and *L. saxatilis.* The "Smooth Periwinkle" of lower levels in the intertidal zone, usually associated with fucoid seaweeds including *Ascophyllum,* is called *Littorina obtusata.* This periwinkle is referred to in modern European physiological literature as *L. littoralis* (L.) (not to be confused with the distinct species *L. littorea* which also occurs in Europe), and in early U. S. conchological literature as *L. palliata* (Say). The "Common Periwinkle" of midlevel intertidal, on most temperate Atlantic shores the largest, most common, and most abundant periwinkle, is universally known as *Littorina littorea.* At higher levels of the intertidal zone around MHWNT is the "Rough Periwinkle" of variable shell form and color, which is here called *Littorina saxatilis.* In most literature, both American and European, *L. rudis* is simply a synonym of *L. saxatilis,* but future studies may divide this polymorphic species.

Lacuna vincta, M. lunata and the three *Littorina* spp. were collected from a boulder beach on Nobska Point (41° 30.9′ N; 70° 39.3′ W) near Woods Hole, Massachusetts on the southern shore of Cape Cod. *A. testudinalis,* along with *Lacuna vincta* and the same three *Littorina* spp., were collected from a boulder beach at Manomet Point, Massachusetts (41° 55.2′ N; 70° 31.2′ W) in the colder waters just north of Cape Cod. During the collection period in the summer of 1973, surface water temperatures ranged from 17.5° C to 25° C at Nobska Point (July 18 through August 12, 1973), giving a mean of 21.6° C, while over the same period mean temperature at Manomet Point was 17.4° C (range = 17.0°–18.0° C).

After each species' collection, the snails were brought to the laboratory in an insulated jar and maintained at ambient water temperature in an incubator in 100% (31.0‰) sea water. All oxygen uptake rate determinations were begun within 72 hours of the time of collection. Oxygen uptake rates in water and in air were monitored with Clark-type polarographic oxygen electrodes (Clark, 1956), purchased from Yellow Springs Instrument Company (Model-53). Respiration chambers were maintained at constant temperatures (± 0.05° C) with a Haake-type Fe constant temperature circulator. The glass respiration chambers (6.9 cm high, 2.04 cm internal diameter) were modified with a glass annulus to contain snails under the magnetic stirrer (diagrams of this and similar modifications are given in Burky, 1977; for use see also: Burky, 1969, 1971; McMahon, 1972, 1973; McMahon and Russell-Hunter, 1977). With this modification the magnetic stirrer spins at approximately 180 revolutions per minute above the snails, and this allows adequate mixing of water in the chamber. The intertidal snails of this study all came from shores which receive moderate amounts of wave action. All six species studied have an adhesive foot and were able to remain actively crawling while attached to the chamber floor and to the walls of the annulus. The currents of the chamber perhaps simulate the water movements experienced by these species in their natural environment. Changes in chamber oxygen concentration were continuously recorded on a Honeywell Electronik-16 Strip Chart Recorder. Aquatic oxygen consumption determinations were made for four to eight subsamples, each of one to ten individuals for the six species studied. The class interval for each subsample was ± 2.5 mm around a chosen shell height, and subsamples were selected to represent the range of shell heights encountered in each field population.

Each experimental subsample was placed in a respiration chamber at ambient field temperature with either 4 or 5 ml of previously boiled and filtered sea water (salinity = 31.0‰, chlorinity = 17.71‰). Temperature was then lowered 3° C every five minutes until 5° C was reached. After equilibration in a blank chamber (with sea water but without snails), the oxygen probe was seated in an experimental chamber and oxygen uptake was continuously monitored from full air saturation (P_{O_2} = 159.1 mm Hg) to an oxygen tension of 90%, or monitored over the reduction in oxygen tension from full air saturation after 30 minutes. This procedure was repeated for all experimental subsamples at 5° C intervals from 5° C through the upper lethal temperature, which varied from 35° C to 45° C depending on species.

During the summer of 1974, oxygen uptake rates from air were determined for *A. testudinalis* from Manomet Point and *L. obtusata, L. littorea* and *L. saxatilis* from Nobska Point. In order to determine aerial respiration rates, the oxygen electrode probe was modified with a neoprene O-ring forming an airtight seal with the chamber wall and by a short length of plastic tubing (1 mm external diameter) to form an air vent to the chamber. The oxygen probe could be inserted in the glass chambers to enclose a sealed volume of 2, 4 or 6 ml of air. (For each species, this volume was less than the corresponding water volume used for measurements of aquatic oxygen uptake.) Aerial oxygen uptake rates were determined for four subsamples of *A. testudinalis*, and for six subsamples each of the three species of *Littorina*. Each subsample contained one to eight individuals depending on size. Snails were blotted on filter paper and placed into the respiration chambers at room temperature (21–23° C). Chamber temperature was then lowered at a rate of 3° C every five minutes until a chamber temperature of 5° C was reached. The oxygen electrode was then equilibrated for 25 minutes in a blank chamber,

TABLE I

Standard weights and corresponding shell heights for the "standard" adult snail in the six intertidal species used. Oxygen uptake values for experimental series (Figures 1–5) were computed by applying these weight values to the 120 regressions of dry tissue weight against oxygen uptake rate, at the appropriate temperatures.

Species	Population	Standard dry tissue weight mg	Corresponding average shell height mm	Number of determinations	Number of regressions	Graphs using this standard
Acmaea testudinalis (Müller)	Manomet	8.3	11.0	90	18	Figs. 1, 2
Mitrella lunata (Say)	Nobska	0.92	4.0	36	7	Fig. 1
Lacuna vincta (Montagu)	Manomet	1.2	3.8	36	7	Fig. 1
	Nobska	1.2	4.3	36	7	Fig. 1
Littorina obtusata (L.)	Manomet	10.0	6.5	72	9	Fig. 3
	Nobska	10.0	7.6	126	18	Fig. 3
Littorina littorea (L.)	Manomet	82.0	14.6	72	9	Fig. 4
	Nobska	82.0	15.4	126	18	Fig. 4
Littorina saxatilis (Olivi)	Manomet	7.8	5.5	72	9	Fig. 5
	Nobska	7.8	6.5	126	18	Fig. 5

before being placed in an experimental chamber, and allowed to equilibate a further 10 minutes with the vent tube open. The vent tube was then clamped shut, and oxygen consumption was then monitored as has been described earlier for aquatic respiration. This procedure was then repeated for all experimental groups at 5° C intervals from 5° C, until each specific upper lethal temperatures was reached. A small piece of silica gel desiccant was placed in both the experimental and blank chambers to obviate water condensation.

After aerial oxygen uptake determinations were complete, the snails in each subsample were blotted on filter paper and their volume and that of the silica gel granule measured by fluid displacement. Each volume of snails plus silica gel was then subtracted from the appropriate chamber volume to give the air volume for that experiment.

After both aquatic and aerial oxygen uptake experiments, the calcareous shells were then removed by treatment with 5% nitric acid for 6 hours. The remaining tissue was rinsed in distilled water and dried to constant weight (95° C for over 12 hours). For each species subsample, oxygen uptake rates were computed as microliters of oxygen consumed per milligram shell-free dry tissue weight per hour [μl O_2/(mg·hr)] at STP. Logarithmic regressions of dry tissue weight *versus* oxygen uptake rate in water and in air at each experimental temperature

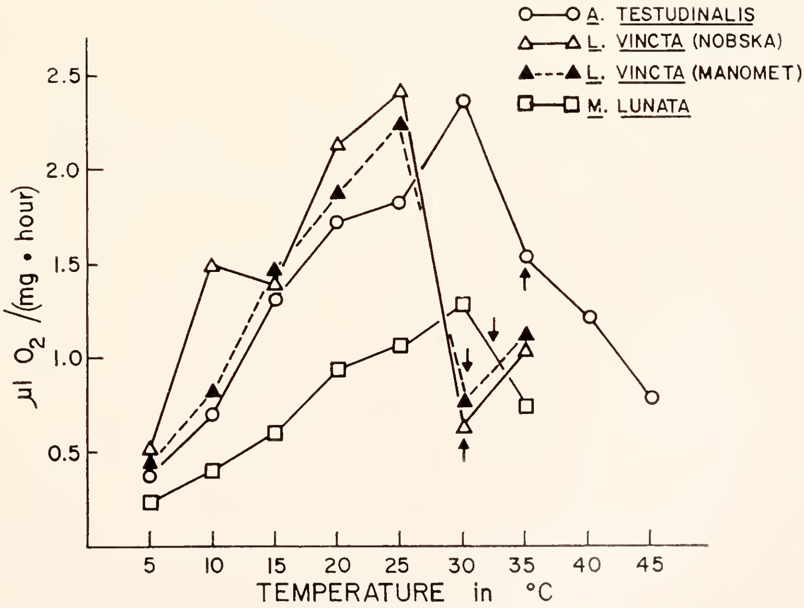


FIGURE 1. The effect of temperature on aquatic oxygen uptake rates in *Acmaea testudinalis*, *Lacuna vincta* and *Mitrella lunata*. The vertical axis represents oxygen uptake rate in microliters of oxygen per milligram shelless dry tissue weight per hour; and the horizontal axis, temperature in degrees centigrade. The open circles represent the oxygen uptake rates of *A. testudinalis* collected from Manomet Point, Massachusetts; the open triangles, that of *L. vincta* from Nobska Point, Massachusetts; the solid triangles, that of *L. vincta* from Manomet Point; and the open squares, that of *M. lunata* from Nobska Point. The vertical arrows indicate approximate lethal temperatures for each experimental group.

were then computed for each species-population tested. (The 120 regression equations are not set out here but can be made available by the senior author, along with the raw data, to any interested investigator.) A species oxygen consumption rate for each experimental temperature was then estimated from these regressions for a standard individual with tissue dry weight representing a modal (median weight) value for a mature adult of that species-population (Table I).

RESULTS

All three subtidal species studied, *Acmaea testudinalis, Lacuna vincta* and *Mitrella lunata,* had similar patterns of respiratory response to increasing temperature. Aquatic oxygen uptake rates in all three species increased steadily from low values at 5° C to peak uptake rates at 25–30° C, which were then immediately followed by a marked decrease in respiration rate at 30–35° C due to thermal stress leading to death (Fig. 1). The Q_{10} of oxygen uptake was very similar for all three species, with *A. testudinalis* having a Q_{10} of 2.05 over 5–30° C. For *M. lunata* Q_{10} was 2.01 over 5–30° C, and for *L. vincta* Q_{10} was 2.15 over 5–25° C for the Nobska Point population, and 2.22 over 5–25° C for those from Manomet Point. Oxygen uptake rates between 5° and 25° C were similar in *A. testudinalis* and *L. vincta,* while *M. lunata* had oxygen rates which were about 50% less up to 25° C (Fig. 1). *L. vincta* from the colder Manomet Point environment (see

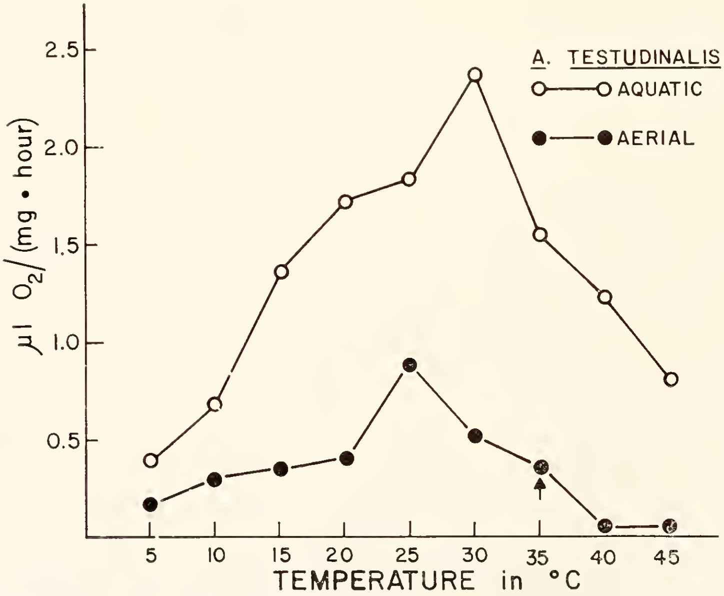


FIGURE 2. The effect of temperature on the aquatic and aerial oxygen uptake rates of *Acmaea testudinalis.* The vertical axis represents oxygen uptake rate in microliters of oxygen per milligram shelless dry tissue weight per hour; and the horizontal axis, temperature in degrees centigrade. The open circles represent the aquatic oxygen uptake rates; and the solid circles, the aerial oxygen uptake rates of *A. testudinalis* collected from Manomet Point. The vertical arrow indicates the approximate lethal temperature.

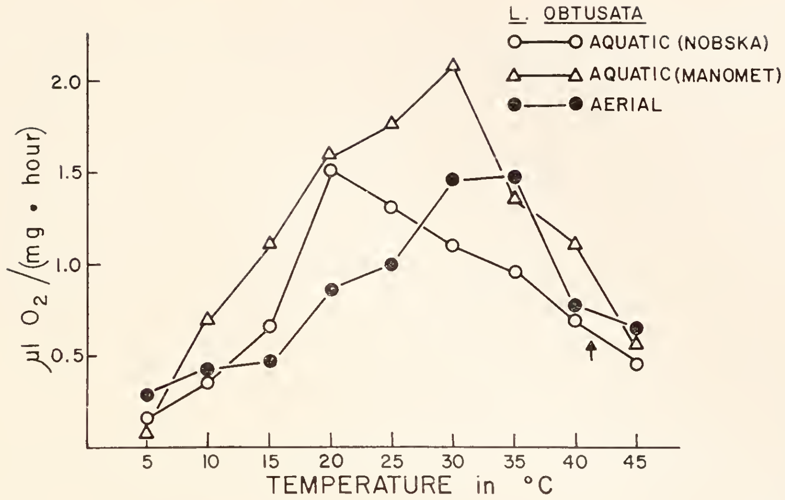


FIGURE 3. The effect of temperature on the aquatic and aerial oxygen uptake rates of *Littorina obtusata*. The vertical axis represents oxygen uptake rate in microliters of oxygen per milligram shelless dry tissue weight per hour; and the horizontal axis, temperature in degrees centigrade. The open circles represent the aquatic oxygen uptake rates; and the solid circles, the aerial oxygen uptake rates of *L. obtusata* collected from Nobska Point. The open triangles represent the aquatic oxygen uptake rates of *L. obtusata* from Manomet Point. The vertical arrow indicates the approximate lethal temperature.

Methods) had a respiratory rate which averaged 12.7% less than that for specimens from Nobska Point through 25° C. Thermal death occurred at about 30° C in *L. vincta* from both the Manomet and Nobska Point populations, at 32.5° C in *M. lunata* and at 35° C in *A. testudinalis* (Fig. 2).

The effects of temperature on the oxygen uptake of *A. testudinalis* in air are similar to those which occur in aquatic respiration. However, oxygen consumption in air is markedly reduced from aquatic rates at all temperatures (Fig. 2) and may reflect the large quantity of mucus secreted over the body surface on exposure of these limpets to air. In air the oxygen consumption of *A. testudinalis* increases from a low rate at 5° C to a peak consumption rate at 25° C. However, the Q_{10} of aerial oxygen uptake is very similar to that recorded for aquatic respiration at 2.20 (Fig. 2). Between 5° and 35° C, aerial oxygen consumption in *A. testudinalis* is only 36.4% of (or 2.75 times less than) aquatic oxygen uptake rates over the same temperature span (Fig. 2).

The patterns of oxygen uptake with increasing temperature in the three congeners, *Littorina obtusata, L. littorea* and *L. saxatilis*, were markedly different from those of the three subtidal species, with each of the three species displaying a uniquely characteristic respiratory response to increasing temperature (Figs. 3–5).

L. obtusata shows a steady increase in aquatic oxygen consumption rates from 5° C up to 20° C in the Nobska Point population, and up to 30° C in the Manomet Point population. The corresponding Q_{10} values are 4.42 (5°–20° C) for the specimens from Nobska Point and 3.18 (5°–30° C) for those from Manomet Point (Fig. 3). Unlike the three sublittoral species, decreases in aquatic oxygen consumption rates from their peak values are not associated with immediate thermal death in *L. obtusata.* Instead, aquatic oxygen uptake rates decrease steadily from either 20° C or 30° C with increasing temperatures until death ensues at about 41.5° C (Fig. 3). The oxygen consumption for specimens of *L. obtusata* from Manomet Point was 47.2% greater than that of specimens from the warmer Nobska Point environment over a temperature range of 5° to 40° C (Fig. 3).

The aerial oxygen uptake in *L. obtusata* from Nobska Point showed a steady increase from 5° to 35° C (Fig. 3). Rates of oxygen consumption in air were similar to, or lower than, aquatic rates for Nobska Point snails between 5°–25° C and higher than aquatic oxygen uptake rates at 30° and 35° C. At 40° C oxygen uptake rates from air were nearly equal to those from water (Fig. 3). For *L. obtusata,* the Q_{10} of oxygen uptake in air was 1.72 over 5°–35° C, a value much lower than the Q_{10} of aquatic oxygen uptake recorded from either the Nobska or Manomet Point populations.

The oxygen uptake of *Littorina littorea* from both water and air was characterized by the maintenance of lower oxygen consumption rates than the other species at all experimental temperatures, and also by an apparent regulation of respiratory rate between 20° and 30° C (Fig. 4). Aquatic oxygen consumption increased from 5° to 20° C in *L. littorea* from Manomet Point with a Q_{10} of 3.63, and from Nobska Point with a Q_{10} of 3.66. However, in both groups, aquatic oxygen uptake showed very little change in rate between 20° and 30° C, with Q_{10} values of 1.04 (Manomet Point) and 0.95 (Nobska Point). At temperatures higher than 35° C, aquatic oxygen consumption decreased steadily in both Nobska and Manomet snails until death occurred at about 43° C. Aquatic rates were very similar in both the Nobska and Manomet Point *L. littorea* populations, with the oxygen uptake rate of Nobska animals an average of only 2.6% greater than that of Manomet animals from 5° to 40° C.

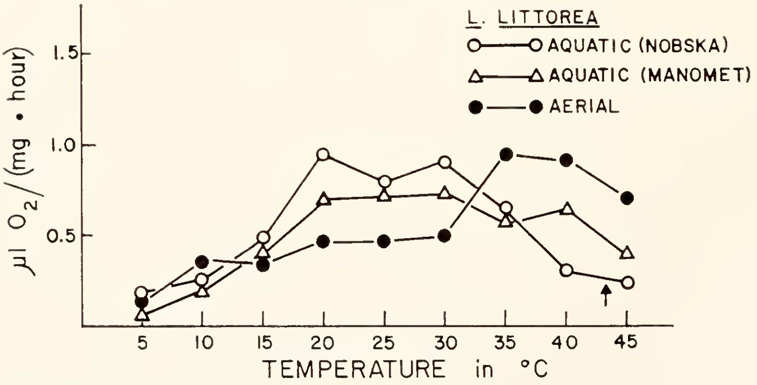


FIGURE 4. The effect of temperature on the aquatic and aerial oxygen uptake rates of *Littorina littorea.* The vertical axis represents oxygen uptake rate in microliters of oxygen per milligram shelless dry tissue weight per hour; and the horizontal axis, temperature in degrees centigrade. The open circles represent the aquatic oxygen uptake rates; and the solid circles, the aerial oxygen uptake rates of *L. littorea* collected from Nobska Point. The open triangles represent the aquatic oxygen uptake rates of *L. littorea* from Manomet Point. The vertical arrow indicates the approximate lethal temperature.

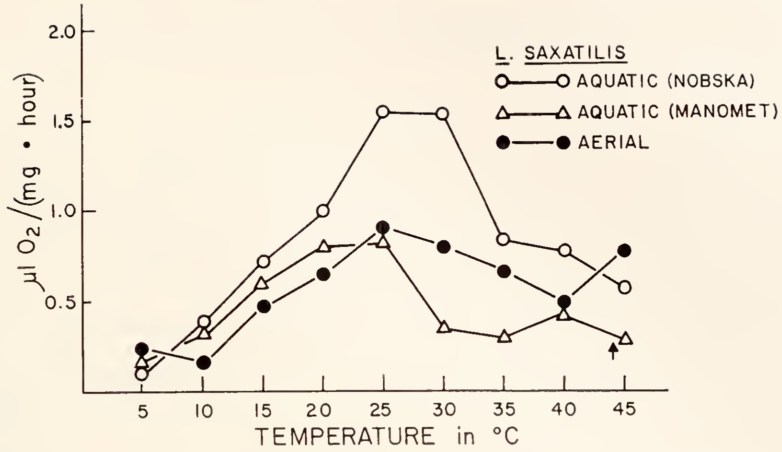


FIGURE 5. The effect of temperature on the aquatic and aerial oxygen uptake rates of *Littorina saxatilis*. The vertical axis represents oxygen uptake rate in microliters of oxygen per milligram shelless dry tissue weight per hour; and the horizontal axis, temperature in degrees centigrade. The open circles represent the aquatic oxygen uptake rates; and the solid circles, the aerial oxygen uptake rates of *L. saxatilis* collected from Nobska Point. The open triangles represent the aquatic oxygen uptake rates of *L. saxatilis* from Manomet Point. The vertical arrow indicates the approximate lethal temperature.

The aerial oxygen uptake of *L. littorea* from Nobska Point showed a similar area of regulation between 20° and 30° C with a Q_{10} of only 1.06. Aerial respiration rate increased between 5° and 20° C with a Q_{10} of 2.68. Unlike aquatic respiration, the aerial oxygen uptake rate of *L. littorea* increased at temperatures higher than 30° C and remained elevated, until death due to thermal stress occurred at about 43° C. In *L. littorea* from Nobska Point, aquatic uptake rates were 47.9% greater between 5° and 30° C than aerial rates, while at 35°–40° C, aerial rates were an average of 125% greater than aquatic rates (Fig. 4).

Littorina saxatilis showed aquatic oxygen uptake rates which increased from 5° to 25° C, with corresponding Q_{10} values of 3.01 (Nobska Point) and 2.49 (Manomet Point) (Fig. 5). Aquatic rates declined sharply at temperatures higher than either 25° or 30° C and remained relatively unchanged, until death occurred at about 44° C. Thus, *L. saxatilis* from Nobska Point had a Q_{10} of 0.85 from 35°–40° C, and Manomet Point animals had a similar Q_{10} of 1.24 over 30°–40° C (Fig. 5). It is of interest to note that the rate decline occurred at 25° C for snails from the cooler Manomet Point habitat, while it occurred at 30° C in *L. saxatilis* from the warmer Nobska Point habitat. At all experimental temperatures, *L. saxatilis* from Nobska Point had uptake rates averaging double those for Manomet Point snails.

Aerial oxygen consumption rates for *Littorina saxatilis* from Nobska Point were generally lower than aquatic rates. However, they showed a pattern of response to increasing temperature which was similar to that for aquatic respiration. In air, rates increased from 5° to 25° C with a Q_{10} of 1.93. At temperatures above 25° C, aerial oxygen consumption decreased continuously until the onset of thermal death at 44° C ($Q_{10} = 0.59$, from 30°–40° C) (Fig. 5). For *L. saxatilis* from Nobska Point, aquatic oxygen consumption rates averaged 57.8% greater than aerial respiration rates from 5° to 25° C.

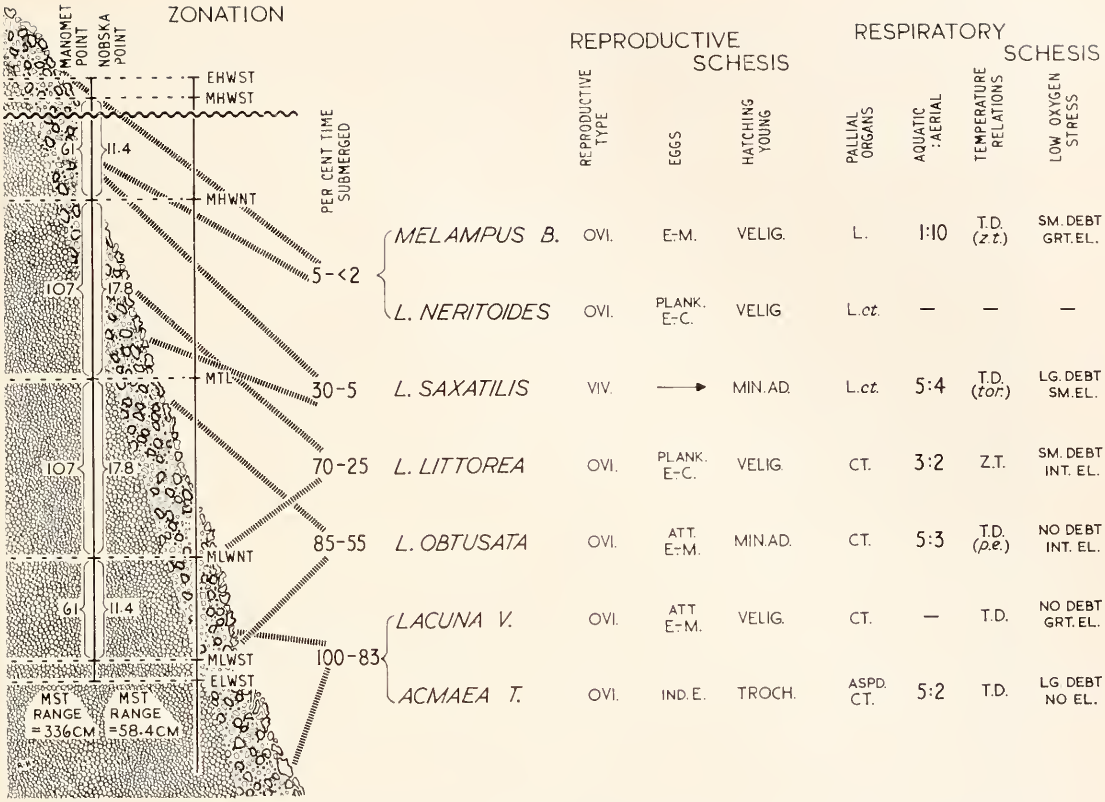
Discussion

The investigation reported in this paper differs from earlier studies on the respiration of intertidal snails in two respects. First, it presents measurements of oxygen uptake made on normally active snails, unlike earlier studies involving a somewhat arbitrarily defined "standard metabolism." Secondly, it is comparative and provides equivalent measures of aquatic oxygen uptake for six, and of aerial uptake for four, different species which live in different vertical zones of the littoral, in contrast to the best of earlier studies (Newell, 1969, 1973; Newell and Pye, 1970a, b, 1971a, b; Pye and Newell, 1973; Newell and Roy, 1973) which are largely limited to one species, *Littorina littorea*. This discussion will briefly consider the relationship between "basal," "standard" and "active" respiratory rates in molluscs such as intertidal snails, before passing to a more extensive consideration of the relationships between patterns of respiratory response to temperature in each species and their distributional ecology, including intertidal vertical zonation.

For many poikilothermic invertebrates, including the majority of littoral and freshwater gastropods, there is no "basal" metabolic rate as the term is understood in vertebrate physiology. In such snails, rates, patterns, and even modes of oxygen uptake are extremely plastic (Russell-Hunter, 1964, 1977). In the earlier work by Newell and his associates (see, for definitions, Newell, 1966; Newell and Pye, 1970b), the difficulty of rates of oxygen uptake varying with degrees of activity was dealt with in the following manner. Regression values of rates against tissue dry weights (least-squares fitting) yielded maximal and minimal curves, which were then termed the "active" rate and the "standard" rate, respectively, and permitted division of the data. In *Littorina littorea*, a zone of thermoregulation of oxygen uptake could only be demonstrated in the minimal or "standard" rate (Newell and Pye, 1970a, b). Subsequently, more sophisticated computations (Newell and Roy, 1973) showed that similar thermoregulation could be claimed for the "active" rates. Of course, many systems both of pallial ciliation and buccal musculature are continuously active in all such gastropods and, under certain conditions in the field, slow locomotion and radular grazing are continuous in many of them. Minimal rates must always be suspect and can only dubiously be claimed as "standard." The investigation reported here concerned normally active snails, and there was no arbitrary division of the data into minimal and maximal groupings; *all* data were utilized in the computation of uptake rates. Snails were chosen to be representative of the size classes (cohorts) present in the natural populations.

Partly as a result of these methodological differences, the work of Newell and his associates is moving towards a more complete elucidation (Newell and Pye, 1970a, b; Newell, 1973) of the cellular and enzymatic bases for respiratory adjustment; while the work reported here (and also on low oxygen stress in the same snails, McMahon and Russell-Hunter, 1974, 1977) allows consideration of interspecific differences and ecological relationships with respiratory measures more closely approximating those for naturally active snails in field populations.

Before the present work, comparative studies on congeneric and other related



PER CENT TIME SUBMERGED		REPRODUCTIVE TYPE	EGGS	HATCHING YOUNG	PALLIAL ORGANS	AQUATIC :AERIAL	TEMPERATURE RELATIONS	LOW OXYGEN STRESS
5-<2	MELAMPUS B.	OVI.	E-M.	VELIG.	L.	1:10	T.D. (*z.t.*)	SM. DEBT GRT. EL.
	L. NERITOIDES	OVI.	PLANK. E-C.	VELIG	L.*ct.*	—	—	—
30-5	L. SAXATILIS	VIV.	⟶	MIN. AD.	L.*ct.*	5:4	T.D. (*tor.*)	LG. DEBT SM. EL.
70-25	L. LITTOREA	OVI.	PLANK. E-C.	VELIG.	CT.	3:2	Z.T.	SM. DEBT INT. EL.
85-55	L. OBTUSATA	OVI.	ATT. E-M.	MIN. AD.	CT.	5:3	T.D. (*p.e.*)	NO DEBT INT. EL.
100-83	LACUNA V.	OVI.	ATT. E-M.	VELIG.	CT.	—	T.D.	NO DEBT GRT. EL.
	ACMAEA T.	OVI.	IND. E.	TROCH.	ASPD. CT.	5:2	T.D.	LG. DEBT NO EL.

FIGURE 6. Vertical zonation in the intertidal with reproductive and respiratory scheses of seven species of littoral snails. Average percentages of time spent submerged are indicated, along with the actual mean spring tide (MST) ranges in centimeters at both Manomet Point and Nobska Point, Massachusetts, U. S. A. The seven species are *Melampus bidentatus*, *Littorina neritoides*, *Littorina saxatilis*, *Littorina littorea*, *Littorina obtusata*, *Lacuna vincta*, and *Acmaea testudinalis*. In the three columns concerned with reproduction, the basic reproductive type is indicated as oviparous (OVI.) or viviparous (VIV.); the egg-laying pattern as individual eggs (IND.E.), egg-capsules (E.-C), or egg-masses (E.-M.) which may be attached (ATT.) or planktonic (PLANK.); and the hatching young as trochophore larvae (TROCH.), veliger larvae (VELIG.) or with the body form of miniature adults (MIN. AD.). In the four columns to the right concerned with respiration, the pallial respiratory organs are designated as having the typical prosobranch gill or pectinibranch ctenidium (CT.), the more primitive "feather-gill" or aspidobranch ctenidium (ASPD. CT.), the mantle-cavity as an air-breathing lung (L.), or as a lung with a vestigial ctenidium (L. *ct.*); the average ratio of aquatic to to aerial oxygen consumption (both assessed at about 22° C) is shown; temperature relations (similar for both aquatic and aerial respiration) are indicated as showing a zone of thermoregulation (Z.T.) or as being largely temperature dependent (T.D.), the latter being modified by some thermoregulation at higher temperatures [T.D. (*z.t.*)], by torpor at higher temperatures [T.D. (*tor.*)], or by involving passive endurance [T.D. (*p.e.*)]; and lastly, the effects of low oxygen stress involve payment of a large (LG.), small (SM.), or NO oxygen debt, and great (GRT.), intermediate (INT.), small (SM.), or NO elevation of post-stressed rates of oxygen uptake at lower (10%) oxygen concentrations. Note that no new data are presented for *Littorina neritoides*, which does *not* occur in our study areas on the mid-Atlantic coast of North America. Although the general adaptational trends from *Acmaea* to *Melampus* are from sea to land, it is clear that the evolution of near-terrestrial structures and functions has proceeded anacoluthically. For further discussion, see text.

intertidal snails were less extensive. Sandison (1966, 1967), and Toulmond (1967a, b) each investigated oxygen consumption in several littoral snails, along the lines of the earlier work of Raffy (1933, see also Fischer, Duval, and Raffy, 1933). Toulmond (1967a, b) worked at a single temperature, and demonstrated that aerial respiration is similar to aquatic in *L. littorea,* close in *L. saxatilis,* but is markedly greater in *L. obtusata.* The earlier French work had reported that aquatic respiration in *L. neritoides* and *L. saxatilis* was several times greater than aerial respiration. Sandison (1966, 1967) claimed that aerial respiration was significantly greater than aquatic respiration in all three species of *Littorina* over a temperature range from 18° C to thermal death (37°–41° C). Obviously, there are contradictory data on the relationship between aquatic and aerial respiration. The results of Raffy and of Toulmond (except for *L. obtusata*) are in agreement with those of the present paper but contradict Sandison's claims. Other earlier reports include excellent comparative studies on desiccation resistance, salinity tolerances, temperature stresses, and levels of thermal death in several series of littoral gastropods in relation to vertical zonation, but do not present any adequate data on respiration rates (Colgan, 1910; Gowanloch and Hayes, 1926; Broekhuysen, 1940; Evans, 1948; North, 1954; Southward, 1958a; Fraenkel, 1960, 1961, 1966, 1968; Lewis, 1963; Markel, 1971).

We have been principally concerned with establishing, for six species of intertidal snails, the patterns of response to temperature change in rates of oxygen uptake. These patterns *do* reflect littoral zonation, particularly the vertical distribution of each species. Three, *Acmaea testudinalis, Lacuna vincta* and *Mitrella lunata,* are essentially sublittoral species which stray into the lower levels of the true littoral and show similar patterns of respiratory response. Aquatic oxygen uptake increases at a constant rate up to 25° or 30° C, and this is followed by a steep decline in uptake leading to thermal death at around 35° C. Aerial uptake, assessed only for *Acmaea,* is lower (between 30% and 50% of aquatic uptake at the same temperatures) but follows the same pattern. In these three species (and probably in the majority of snail species living subtidally), there is no obvious metabolic adjustment to higher temperatures. The other species, *Littorina obtusata, L. littorea* and *L. saxatilis,* are true intertidal snails and, despite some overlaps, the three congeners show a clear vertical zonation. In *L. obtusata,* the "lowest" of the three, both aerial and aquatic uptake rates increase steadily within the normal temperature range (up to about 25° C), then decline slowly (in a "passive endurance," quite unlike the responses of the sublittoral species). In the dominant snail of the midlittoral, *Littorina littorea,* aerial and aquatic respiration are rather similar up to 30° C (corresponding to normal upper limits for their environment). Unlike *Acmaea* and *L. obtusata,* increase in uptake for *L. littorea* between 15° C and 30° C is considerably less than between 5° C and 15° C (or, for that matter, above 30° C), constituting a zone of metabolic regulation, in both aerial and aquatic respiration. Finally, *Littorina saxatilis* shows somewhat lower aerial than aquatic respiration rates at all temperatures. The lower rates for *L. saxatilis* above 25° C occur well below temperature levels at which thermal deterioration sets in. These lower rates represent a reversible "torpor" or short-term diapause, which fits with the specific habitat (70%–95% of their time out of water). It is worth noting that these major shifts, with changes of temperature, and between aerial and aquatic respiration, are many times greater than the rhythmic metabolic shifts known to occur under near-constant conditions (see, for example, Sandeen, Stephens and Brown, 1954).

In discussing the ecological significance of these respiratory patterns, it is appropriate to assess for each level of habitat the mean proportion of time in each semilunar tidal cycle of 354.4 hours when that particular zone is actually bathed by sea water. In Figure 6 are shown the average percentages of time spent submerged, as well as the vertical distributions of the species and the actual tidal ranges involved at Nobska and Manomet Points. *Mitrella lunata*, being essentially similar to *Acmaea* and *Lacuna* is omitted from Figure 6, while two species of the highest levels of the littoral are included for comparison: a fourth congener, *Littorina neritoides* (which does *not* occur on the mid-Atlantic coasts of North America, and for which we have no new data), and the primitive pulmonate of high-level salt-marshes, *Melampus bidentatus* (for which we have some respiratory and survivorship data, McMahon and Russell-Hunter, 1975). Figure 6 also presents an attempt to summarize certain significant aspects of both the reproductive and respiratory scheses of these seven snails. Taken as a whole, the general adaptational trends run from sea to land—from *Acmaea* to *Melampus*—with many irregularities intervening in each functional series. With the obvious exception of the serial shift from an aspidobranch ctenidium (a gill suitable only for clean waters) to a lung, the evolution of near-terrestrial structures and functions has proceeded anacoluthically. Similarly discontinuous series (not presented here) would involve water-control and nitrogenous excretion in these snails. Although doubts have arisen regarding the much-quoted figures on the uric content of gastropods which were first estimated by Needham (1935, 1938; see also Spitzer, 1937; Campbell, 1973), retention of uric acid (though perhaps not of a predominantly uricotelic metabolism paralleling that of reptiles and birds) is apparently greater in *Littorina neritoides* and *L. saxatilis* than it is in *L. obtusata*, while it is least in *L. littorea*. The "higher" species of *Littorina* have a greater capacity to resist water-loss but, under desiccating conditions, rates of body water loss in *Melampus bidentatus* are drastically high. This species is characterized not only by tissues extremely tolerant of desiccation and a capacity for rapid rehydration, but also by a repertoire of behavioral patterns which tend to "hold" populations in more humid microclimates (Price and Russell-Hunter, 1975; Russell-Hunter and Meadows, 1965).

Despite the discontinuities in most series, the broad ecological "rules" of littoral zonation, as elucidated by the Stephensons and by Southward (Stephenson and Stephenson, 1949, 1972; Southward, 1958b), including the prediction that no species thriving below MLWST can extend its range above MTL, hold for our snails. As often set out, environmental conditions just below MLWST are not greatly different from those in the depths of the ocean, while animals living above MHWNT are practially terrestrial. It is important to distinguish between the rather rigid vertical zonation of sessile animals such as barnacles and that of mobile animals such as our snails, where migratory behavior can modify distribution patterns on a shorter or longer time-scale. As discussed some years ago by G. E. Newell (Newell, 1954, 1958a, b; Smith and Newell, 1955; see also Newell, 1964), populations of *Littorina* spp. show both seasonal and shorter-term vertical shifts on the shore. Another significant factor concerns the considerable differences in dispersal ability which arise from differences in the eggs and larvae (see mid-columns of Figure 6). As a result of electrophoretic studies on polymorphic esterases in New England and Canadian Atlantic populations, Berger (1973) has concluded that marked differences in such gene frequencies in the three common *Littorina* species are correlated with the extent of larval dispersal (there being significantly less local geographic differentiation in *L. littorea* than in the other two species).

To recapitulate, the patterns of aerial and aquatic respiration described here reflect vertical zonation. The patterns shown by *Littorina obtusata* differ but little from those in the three sublittoral species, though *L. obtusata* shows a greater tolerance of high temperatures and a more "balanced" aerial respiration. The case of *Littorina littorea* is of particular interest. Its great success as the dominant snail of the midlittoral depends, among other things, upon its capacity to maintain a degree of regulation in its aquatic respiration over the more usual environmental temperature range (17° C–32° C). This capacity for regulation in aquatic oxygen uptake appears to dictate the pattern of its aerial respiration (see Fig. 4), and may have prevented its developing any pattern of diapause which would be more appropriate to the greater range of air temperatures in the upper littoral. Such a pattern (involving a reversible torpor under high temperatures) *does* characterize the respiratory schesis of *Littorina saxatilis,* ensconced at higher tidal levels. Lastly, *Melampus* is primarily an air-breather with only a limited capacity for aquatic respiration at higher temperatures (McMahon and Russell-Hunter, 1975).

As discussed elsewhere (McMahon and Russell-Hunter, 1974, 1977), interspecific differences in these snails in responses to low oxygen stress are less related to vertical zonation, but rather to microenvironmental features and to the physiological ecology of each individual species. In contrast, in these temperature relations of oxygen consumption, interspecific—and even some infraspecific—differences are more directly related to degrees of submergence/exposure, and hence to the vertical distribution patterns of the snails on the seashore.

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In addition, one of us (W.D.R-H.) wishes to take this opportunity to recall with gratitude his own first introduction by two different biologists (three decades ago at Millport in Scotland) to the fascination of intertidal snails. My field introduction to littoral zonation was by Richard Elmhirst, an extraordinary naturalist who published little but freely communicated much of his extensive knowledge of the living animals of the shore, and who died in 1948; while my introduction to the physiological implications of littoral life, particularly among snails, came from C. M. Yonge (now Sir Maurice Yonge) who is still, happily, engaged in his worldwide studies elucidating function and form in marine molluscs. They both contributed much to the background of this and other papers.

SUMMARY

Aerial and aquatic rates of oxygen consumption were determined over a range of 5° to 45° C at 5° C intervals for six species of marine littoral snails: including

the sublittoral species, *Acmaea testudinalis, Mitrella lunata,* and *Lacuna vincta;* and the truly intertidal species, *Littorina obtusata, L. littorea,* and *L. saxatilis.* Polarographic oxygen electrodes were used with normally active snails collected from populations on Nobska and Manomet Points, Massachusetts.

Three subtidal species, *A. testudinalis, Lacuna vincta,* and *M. lunata,* do not display any metabolic adjustment to increasing temperature, with thermal limits reached at 30° to 35° C. Aerial respiration in *A. testudinalis* is similar to aquatic O_2 uptake, but rates average only 36.4% of aquatic rates.

The intertidal congeners, *Littorina obtusata, L. littorea* and *L. saxatilis,* have varying degrees of aerial and aquatic metabolic regulation with increasing temperature. *L. obtusata,* a low intertidal snail exposed to air for 15% to 45% of the tidal cycle, displays a respiratory pattern of "passive endurance" to high temperatures both in air and in water. *L. littorea,* the dominant snail of the mid-littoral region, remains active when exposed to air (30% to 75% of the tidal cycle) and has a zone of metabolic regulation between 20° C and 30° C. Over this, the normal ambient temperature range, the Q_{10} closely approximates one, and nearly equivalent O_2 uptake rates occur in air and in water. *L. saxatilis* from the upper littoral region is exposed to air for 70% to 95% of the tidal cycle and is characterized by reduced aerial and aquatic O_2 uptake rates above 25° C, representing a reversible torpor up to its thermal maximum at 44° C.

For these six snail species, respiratory responses to increasing temperature are thus directly related to the pattern of vertical distribution in the intertidal environment. Discussion of this relationship stresses that the evolution of other near-terrestrial structures and functions in littoral snails has proceeded in a discontinuous fashion. Despite this, the temperature responses in respiration parallel the functional morphology of the pallial structures and the physiological patterns of response to low oxygen stress, as well as adaptive features of reproduction, larval development, water-control, and nitrogenous excretion.

NOTE ADDED IN PRESS

Difficulties in the systematic status of one of our six species, *Littorina saxatilis,* have long been appreciated, but it has usually been regarded as constituting a single but polymorphic species. While both the present paper and another on the effects of low oxygen stress on the respiration of littoral snails (McMahon and Russell-Hunter, 1977) were being revised for publication, two important contributions on *Littorina* spp., one British (Heller, 1975) and one Russian (Mileikovsky, 1975), became available. On the basis of a variety of evidence, including different allozyme patterns for esterases from disc electrophoreses and differences in penial anatomy, Heller (1975) has concluded that his natural populations of the "*saxatilis*" aggregate in Wales consist of four separate but essentially sympatric species. Whatever is subsequently elucidated regarding the taxonomy of the species currently called *Littorina saxatilis* on the Atlantic coast of the United States, we had safeguarded the identification of the material used in both of our respiratory studies by depositing (while the work was still in progress, 1973–74) voucher specimens of our "*L. saxatilis*" in the collections established by Professor R. Tucker Abbott for this purpose at the Delaware Museum of Natural History. Both Heller (1975) and Mileikovsky (1975) also discuss evidence that certain forms in the "*saxatilis*" aggregate are viviparous and others oviparous. After a survey of 39 species of littorinids throughout the world, Mileikovsky concludes that any oversimple scheme representing these snails as "text-book" examples of a supposed obligate relationship between pattern of larval development and intertidal zonation "must be rejected." If future work should show that the populations currently termed *Littorina saxatilis* on the U. S. Atlantic coast include both viviparous and oviparous forms, the necessary modification of the reproductive data summarized here need not alter the general conclusion (see Figure 6, and discussion on above) that the evolution of near-terrestrial structures and functions in littoral snails has proceeded in a discontinuous fashion.

LITERATURE CITED

Berger, E. M., 1973. Gene-enzyme variation in three sympatric species of *Littorina*. *Biol. Bull.*, **145**: 83–90.

Broekhuysen, G. J., 1940. A preliminary investigation of the importance of desiccation, temperature, and salinity as factors controlling the vertical distribution of certain intertidal marine gastropods in False Bay, South Africa. *Trans. R. Soc. S. Afr.*, **28**: 255–291.

Burky, A. J., 1969. Biomass turnover, energy balance, and interpopulation variation in the stream limpet, *Ferrissia rivularis* (Say), with special reference to respiration, growth, and fecundity. *Ph.D. thesis, Syracuse University*, Syracuse, New York, 104 pp. (*Diss. Abstr.*, **30**: 3917–B; order no. 70–1942).

Burky, A. J., 1971. Biomass turnover, respiration and interpopulation variation in the stream limpet, *Ferrissia rivularis* (Say). *Ecol. Monogr.*, **41**: 235–251.

Burky, A. J., 1977. Respiration chambers for measuring oxygen consumption of small aquatic molluscs with Clark-type polarographic electrodes. *Malacol. Rev.*, **10**: in press.

Campbell, J. W., 1973. Nitrogen excretion. Pages 279–316 in C. L. Prosser, Ed., *Comparative animal physiology*. 3rd Ed. W. B. Saunders Company, Philadelphia.

Colgan, N., 1910. Notes on the adaptability of certain littoral mollusca. *Ir. Nat. J.*, **19**: 127–133.

Evans, R. G., 1948. The lethal temperatures of some common British littoral molluscs. *J. Anim. Ecol.*, **17**: 165–173.

Fischer, P.-H., M. Duval, and A. Raffy, 1933. Études sur les échanges respiratoires des littorines. *Arch. Zool. Exp. Gen.*, **74**: 627–634.

Fraenkel, G., 1960. Lethal high temperatures for three marine invertebrates: *Limulus polyphemus, Littorina littorea* and *Pagurus longicarpus*. *Oikos*, **11**: 171–182.

Fraenkel, G., 1961. Resistance to high temperatures in a Mediterranean snail, *Littorina neritoides*. *Ecology*, **42**: 604–606.

Fraenkel, G., 1966. The heat resistance of intertidal snails at Shirahama, Wakayama-ken, Japan. *Publ. Seto Mar. Biol. Lab.*, **14**: 185–195.

Fraenkel, G., 1968. The heat resistance of intertidal snails at Bimini, Bahamas; Ocean Springs, Mississippi; and Woods Hole, Massachusetts. *Physiol. Zool.*, **41**: 1–13.

Gowanloch, J. N., and F. R. Hayes, 1926. Contributions to the study of marine gastropods. I. The physical factors, behaviour, and intertidal life of *Littorina*. *Contrib. Can. Biol. Fish.*, **3**: 133–165 (plus plates).

Heller, J., 1975. The taxonomy of some British *Littorina* species, with notes on their reproduction (Mollusca: Prosobranchia). *Zool. J. Linn. Soc.*, **56**: 131–151.

Lewis, J. B., 1963. Environmental and tissue temperatures of some tropical intertidal marine animals. *Biol. Bull.*, **124**: 277–284.

Markel, R. P., 1971. Temperature relations in two species of tropical West American littorines. *Ecology*, **52**: 1126–1130.

McMahon, R. F., 1972. Interpopulation variation and respiratory acclimation in the bioenergetics of *Laevapex fuscus*. *Ph.D. thesis, Syracuse University*, Syracuse, New York, 163 pp. (*Diss. Abstr.*, **33**: 5068–B; order no. 73–9549).

McMahon, R. F., 1973. Respiratory variation and acclimation in the freshwater limpet, *Laevapex fuscus*. *Biol. Bull.*, **145**: 492–508.

McMahon, R. F., and W. D. Russell-Hunter, 1973. Respiratory adaptability in relation to vertical zonation in littoral and sublittoral snails. *Biol. Bull.*, **145**: 447.

McMahon, R. F., and W. D. Russell-Hunter, 1974. Responses to low oxygen stress in relation to the ecology of littoral and sublittoral snails. *Biol. Bull.*, **147**: 490.

McMahon, R. F., and W. D. Russell-Hunter, 1975. Temperature and salinity effects on *Melampus bidentatus* Say with special reference to oxygen uptake rates and survival. *Biol. Bull.*, **149**: 436.

McMahon, R. F., and W. D. Russell-Hunter, 1977. Physiological responses to low oxygen stress in marine littoral and sublittoral snails and the diversity of their environmental relationships. Submitted.

Mileikovsky, S. A., 1975. Types of larval development in Littorinidae (Gastropoda: Prosobranchia) of the world ocean, and ecological patterns of their distribution. *Mar. Biol. (Berl.)*, **30**: 129–135.

Needham, J., 1935. Problems of nitrogen catabolism in invertebrates. II. Correlation between uricotelic metabolism and habitat in the phylum Mollusca. *Biochem. J.*, **29**: 238–251.

Needham, J., 1938. Contributions of chemical physiology to the problem of reversibility in evolution. *Biol. Rev.*, **13**: 225–251.

Newell, G. E., 1954. Animal zones of the North Kent coast. *South Eastern Naturalist and Antiquary*, **49**: 34–54.

Newell, G. E., 1958a. The behaviour of *Littorina littorea* (L.) under natural conditions and its relation to position on the shore. *J. Mar. Biol. Assoc. U. K.*, **37**: 229–239.

Newell, G. E., 1958b. An experimental analysis of the behaviour of *Littorina littorea* (L.) under natural conditions and in the laboratory. *J. Mar. Biol. Assoc. U. K.*, **37**: 241–266.

Newell, G. E., 1964. Physiological aspects of the ecology of intertidal molluscs. Pages 59–81 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of Mollusca, Vol. I.* Academic Press, New York and London.

Newell, R. C., 1966. The effect of temperature on the metabolism of poikilotherms. *Nature*, **212**: 426–428.

Newell, R. C., 1969. The effect of temperature fluctuation on the metabolism of intertidal invertebrates. *Amer. Zool.*, **9**: 293–307.

Newell, R. C., 1973. Factors affecting the respiration of intertidal invertebrates. *Amer. Zool.*, **13**: 513–528.

Newell, R. C., and V. I. Pye, 1970a. Seasonal changes in the effect of temperature on the oxygen consumption of the winkle *Littorina littorea* (L.) and the mussel *Mytilus edulis* L. *Comp. Biochem. Physiol.*, **34**: 367–383.

Newell, R. C., and V. I. Pye, 1970b. The influence of thermal acclimation on the relation between oxygen consumption and temperature in *Littorina littorea* (L.) and *Mytilus edulis* L. *Comp. Biochem. Physiol.*, **34**: 385–397.

Newell, R. C., and V. I. Pye, 1971a. Quantitative aspects of the relationship between metabolism and temperature in the winkle *Littorina littorea* (L.). *Comp. Biochem. Physiol.*, **38B**: 635–650.

Newell, R. C., and V. I. Pye, 1971b. Temperature induced variations in the respiration of mitochondria from the winkle *Littorina littorea* (L.). *Comp. Biochem. Physiol.*, **40B**: 249–261.

Newell, R. C., and A. Roy, 1973. A statistical model relating the oxygen consumption of a mollusk (*Littorina littorea*) to activity, body size, and environmental conditions. *Physiol. Zool.*, **46**: 253–275.

North, W. J., 1954. Size distribution, erosive activities, and gross metabolic efficiency of the the marine intertidal snails, *Littorina planaxis* and *L. scutulata*. *Biol. Bull.*, **106**: 185–197.

Price, C. H., and W. D. Russell-Hunter, 1975. Behavioral and physiological aspects of water relations in the high littoral snail, *Melampus bidentatus* Say. *Biol. Bull.*, **149**: 442–443.

Pye, V. I., and R. C. Newell, 1973. Factors affecting thermal compensation in the oxidative metabolism of the winkle *Littorina littorea*. *Neth. J. Sea Res.*, **7**: 411–420.

Raffy, A., 1933. Recherches sur le métabolisme respiratoire des poïkilothermes aquatiques. *Ann. Inst. Océanogr. Monaco*, **13**: 263–393.

Russell-Hunter, W., 1964. Physiological aspects of ecology in nonmarine molluscs. Pages 83–126 in K. M. Wilbur and C. M. Yonge, Eds., *Physiology of Mollusca, Vol. I.* Academic Press, New York and London.

Russell-Hunter, W. D., 1977. Ecology of freshwater pulmonates. Pages 000–000 in V. Fretter and J. F. Peake, Eds., *Pulmonates, Volume 2: Systematics, evolution and ecology.* Academic Press, New York and London.

Russell-Hunter, W. D., and R. F. McMahon, 1974. Patterns of aerial and aquatic respiration in relation to vertical zonation in four littoral snails. *Biol. Bull.,* **147**: 496–497.

Russell-Hunter, W. D., and R. T. Meadows, 1965. Aspects of water physiology in the the salt-marsh pulmonate snail, *Melampus bidentatus. Biol. Bull.,* **129**: 409.

Sandeen, M. I., G. C. Stephens, and F. A. Brown, Jr., 1954. Persistent daily and tidal rhythms of oxygen consumption in two species of marine snails. *Physiol. Zool.,* **27**: 350–356.

Sandison, E. E., 1966. The oxygen consumption of some intertidal gastropods in relation to zonation. *J. Zool. (Lond.),* **149**: 163–173.

Sandison, E. E., 1967. Respiratory response to temperature and temperature tolerance of some intertidal gastropods. *J. Exp. Mar. Biol. Ecol.,* **1**: 271–281.

Smith, J. E., and G. E. Newell, 1955. The dynamics of the zonation of the common periwinkle (*Littorina littorea* (L.)), on a stony beach. *J. Anim. Ecol.,* **24**: 35–56.

Southward, A. J., 1958a. Note on the temperature tolerances of some intertidal animals in relation to environmental temperatures and geographical distribution. *J. Mar. Biol. Assoc. U. K.,* **37**: 49–66.

Southward, A. J., 1958b. The zonation of plants and animals of rocky sea shores. *Biol. Rev.,* **33**: 137–177.

Spitzer, J. M., 1937. Physiologisch-ökologische Untersuchungen über den Exkretstoffwechsel der Mollusken. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere,* **57**: 457–496.

Stephenson, T. A., and A. Stephenson, 1949. The universal features of zonation between tide-marks on rocky coasts. *J. Ecol.,* **37**: 289–305.

Stephenson, T. A., and A. Stephenson, 1972. *Life between tidemarks on rocky shores.* W. H. Freeman and Company, San Francisco, 425 pp.

Toulmond, M. A., 1967a. Étude de la consommation d'oxygène en fonction du poids, dans l'air et dans l'eau, chez quatre espèces du genre *Littorina* (Gastropoda, Prosobranchiata). *C. R. Hebd. Seances Acad. Sci. Paris,* **264**: 636–638.

Toulmond, A., 1967b. Consommation d'oxygène, dans l'air et dans l'eau, chez quatre Gastéropodes du genre *Littorina. J. Physiol. (Paris),* **59(1)**: 303–304.

Reference: *Biol. Bull.*, **152**: 199–208. (April, 1977)

EFFECTS OF A JUVENILE HORMONE MIMIC ON MALE AND FEMALE GAMETOGENESIS OF THE MUD-CRAB, *RHITHROPANOPEUS HARRISII* (GOULD) (BRACHYURA: XANTHIDAE)

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Juvenile hormone (JH) analogs are substances of natural and synthetic origin which act in the same way as endogenous JH, the endocrine secretion of the insect *corpora allata*. The wide range of their biological effects is now well established and has led to their study as a possible means of pest control (reviews by Novák, 1971; Stockel, 1975). In consequence, nontarget organisms may also be contaminated through the food-chain or by residues of the exogenous hormone degradation; likewise, JH analogs may enter the aquatic surroundings with land-drainage or erosion from the adjacent pesticide-treated agricultural lands and tidal marshes. Considering the potential role played by JH analogs as insecticides, it seems of particular interest to determine whether these compounds may affect crustaceans, generally accepted to be closely related to insects.

JH mimics are known to disrupt embryonic and larval insect development and to induce sterility in adults (reviews by Bowers, 1971; Sláma, 1971; Sláma, Romaňuk and Šorm, 1974), rather than to cause immediate death. However, the results depend largely on the type of analog, the dose and time of application and also the optimal sensitive period of the animal. Although there have not been sufficient investigations to demonstrate the existence of crustacean organs similar to the insect *corpora allata*, one must take into consideration the recent ultrastructural study by Byard and Shivers (1975), which suggests a possible analogy with decapod mandibular organs. In line with this hypothesis, decapods thus appear to be a good choice to search for target organs which can respond to insect JH. Knowledge of the normal reproductive physiology of the estuarine mud-crab *Rhithropanopeus harrisii* (Payen, 1974b) and the availability of one JH mimic (Altosid®) prompted this investigation, to determine in what way male and female gametogenesis in an estuarine crab may be affected under laboratory conditions.

Very few studies have been devoted to the influence of natural or synthetic JH on crustaceans. A physiological effect showing vitellogenic inhibition has been observed in the amphipod *Orchestia gammarellus* (Charniaux-Cotton, 1974) after injection of synthetic JH-I. Two morphological effects are actually known. One is related to the blocking of egg development in the isopod *Armadillidium vulgare* (Greer, cited in Pihan, 1975) with topical application of both analogs and natural JH-I; the other demonstrates a precocious metamorphosis without settlement in the acorn barnacle, *Balanus galeatus,* reared with the synthetic analog,

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ZR-512 (Gomez, Faulkner, Newman and Ireland, 1973) or with freshly synthesized JH-I (Ramenofsky, Faulkner and Ireland, 1974). A third study (Costlow, 1976) has shown that a similar compound, methoprene (Altosid®: ZR-515), while not affecting metamorphosis itself, does result in reduced survival of larvae of the mud-crab, *Rhithropanopeus harrisii;* there is evidence of synergistic effects of salinity and methoprene, and also that the early megalopa, as opposed to the zoeal stages, represents a developmental stage of extreme sensitivity.

MATERIAL AND METHODS

Hormonal material

Numerous chemical compounds with juvenilizing activity, also called juvenoids or insect growth regulators, have been synthesized during the past few years (reviews by Sláma, Romaňuk and Šorm, 1974; Pihan 1975). We used methoprene (made by the Zoëcon Corporation, Palo Alto, California), or isopropyl-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate or Altosid® (ZR-515), which is relatively stable. Its efficacy has been proved in different insects: *Galleria melonella, Aedes aegypti, Tenebrio molitor, Schistocerca gregaria, Culex tarsalis, Gryllus bimaculatus* and *Musca domestica* (Henrick, Staal, Siddal, 1973; Reddy and Krishnakumaran, 1973; Roussel and Perron, 1974; Ittycheriah, Marks and Quraishi, 1974; Crochard, 1975; Yu and Terriere, 1975). This $C_{19}H_{34}O_3$ compound is a mixture of isomers containing 69.0% *trans trans* form, closely related

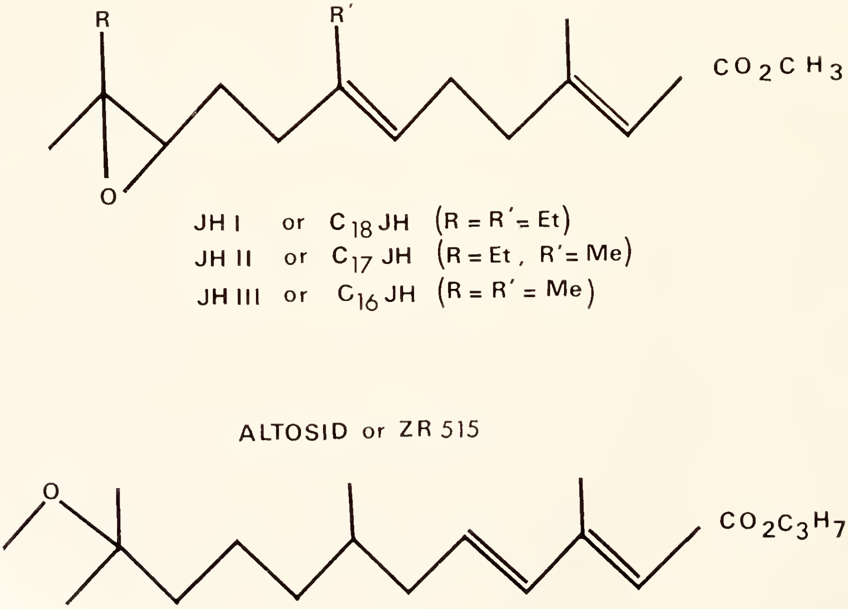
R
R′
CO2CH3
O
JH I or C18 JH (R = R′= Et)
JH II or C17 JH (R = Et , R′= Me)
JH III or C16 JH (R = R′ = Me)
ALTOSID or ZR 515
O
CO2C3H7

FIGURE 1. Comparative chemical structures of the acids of the three juvenile hormones known in insects (JH-I, JH-II, JH-III) and one analog, methoprene (Altosid® or ZR-515). *Cis* and *trans* configurations are omitted in the diagrams.

to the three juvenile hormones (JH-I, JH-II, JH-III) now known to occur in nature (Fig. 1). Its chromatographic properties have been recently analyzed by Dunham, Schooley and Siddall (1975).

Experimental animals

In August 1974, 28 mature specimens of *Rhithropanopeus harrisii* (Gould) (14 females, 14 males) with a cephalothoracic length varying between 5 and 8 mm were collected in the Neuse River estuary at a place called "Pine Cliff" near Havelock, North Carolina, and kept isolated.

The experimental series included seven male and seven female crabs which were reared in 1.30 ppm Altosid® (90.7% active ingredient) in 20‰ sea water at 25° C. Methoprene was dissolved in 100 ml of full strength insecticide grade acetone to make a stock solution. A dilution of 1.30 ppm was made by adding 1 ml of the stock solution to 999 ml of 20‰ sea water. This concentration is close to the limit (1.39 ppm) of solubility of methoprene and allows one to determine the effect of the highest concentration of the compound.

There were two control series: a seawater control with three crabs of each sex and an acetone control with four crabs of each sex. The seawater control had a salinity of 20‰, and the acetone control was made up by adding 1 ml of full strength acetone to 999 ml of sea water with a salinity of 20‰. Thus, the acetone control and the experimental medium had the same concentration of 1 ppt acetone. The crabs were changed every three days to clean bowls containing fresh media and fed with four day old *Artemia salina* nauplii.

No morphological or physiological differences were noted between the crabs reared in seawater control and acetone control. Three females laid eggs in these media, but none were laid in the experimental one. The whole experiment was conducted for one and a half months.

Methoprene activity was assayed on *R. harrisii* spermatogenesis and oogenesis following a double method of application: ingestion and topical application in the ambient aquatic environment. This process is actually considered to be less toxic and more active than injections (Sláma, Romaňuk and Sŏrm, 1974, pp. 98–104).

Histological procedure

Testes and ovaries were examined both anatomically and histologically. Whole mounts and 5 μm sections of treated and control gonads were prepared after being removed from the crabs and fixed in Duboscq Brasil's solution. Alum-carmine and haematoxylin-picro-indigo carmine were the primary stains. Study of *R. harrisii* gonads was undertaken on crabs 4.7 to 8.0 mm cephalothoracic length. To follow the appearance of possible abnormalities or gametogenic perturbations which take place after methoprene treatment, the animals were sacrificed after 12 and 45 days.

RESULTS

Although no particular relationship between physiological processes such as molting and gametogenesis is known, it seems appropriate to include the following observations for future studies.

TABLE I

Summary of seven male and seven female experimental crabs, their molt status, and times of exposure to methoprene.

Status of crabs at examination			Number of days of exposure to methoprene (1.39 ppm)	Size: cephalothoracic length (mm)	Effects on gametogenesis
Without molting	Just after a lethal molt	Three days after a normal molt			
♂			7	5.0	stimulation of spermatogenesis
♂			9	6.2	
♂			12	7.0	
♂			24	8.0	inhibition of spermatogenesis
	♂		43	7.2	
	♂		43	4.9	
		♂	45	8.0	
♀			15	5.3	no effect
♀			15	6.6	inhibition of vitellogenesis
♀			18	6.9	
	♀		38	6.8	
	♀		41	7.0	
	♀		42	7.5	
♀			45	7.3	

Two females and one male out of six reared in sea water molted during the experimental period. Among those maintained in the sea water-acetone medium, one male and two females molted normally, whereas one female died 18 hours after exuviation. Crabs from the experimental series (sea water plus methoprene-acetone solution) showed a high rate of mortality at the time of ecdysis which always appeared delayed (between 38 to 43 days of treatment) compared to controls of the same size (average of eight days after collecting). Only one male survived after shedding its exuvia, whereas this phase was lethal for two other males and three females. Nonachievement of the molting process thus appears to be due to the combined effects of both methoprene and acetone, which may produce a dissolving action through the new formed integument. Table I summarizes the data obtained.

Effect of Allosid® on R. harrisii *spermatogenesis*

In previous work (Payen, 1974a), a correlation between the encasement of gonopods (Pl_2 becomes inserted into a foramen of Pl_1 instead of remaining free and independent) and the onset of spermatogenesis was found, allowing a quick evaluation of the state of the gonads, especially in young males. Only crabs with encased gonopods were considered. After methoprene treatment, they all exhibited a more developed genital apparatus than that of controls with the same cephalothoracic length but were less developed than crabs destalked during larval life and sacrificed at juvenile stages (Payen, Costlow and Charniaux-Cotton, 1971).

In particular, the androgenic glands, which are known to be affected by destalking experiments (Payen, 1974b), always keep a volume and a structure similar to those of control crabs. During the first twelve days, all stages of spermatogenesis are well recognizable; they take place in more numerous and prominent convolutions than in testes of animals reared only in sea water. Moreover, the genital ducts are swollen and full of homogenous acidophilic secretions. In males sacrificed between 18 and 45 days, the testes appear stunted and become difficult to dissect (60 μm of average diameter as opposed to 120 μm in males of the same size reared in seawater control). Spermatogenesis is considerably reduced; most of the testicular volume is taken up by rare spermatocytes in meiotic prophase, few spermatids and many spermatozoa which are drained off in the overstretched spermducts. Gonia disappear, and the few which are left do not show mitotic figures as in control testes; some are pycnotic. Since cell divisions are approximately synchronous within each testicular lobe, it is easy to note the amalgam of spermatocyte clusters which constitutes degenerative glomerules reaching a diameter of about 15 μm; these compact formations are more or less scattered or abundant, depending on time of sacrifice. Thus, the ultimate step of degeneration leads to the complete lysis of the testicular lobes. It then appears that the methoprene-exposed crabs undergo a transitory increase of spermatogenesis (during the first 12 days of contamination), characterized by a massive accumulation of spermatozoa in the *vasa deferentia*. Later, no new spermatogenesis occurs, because spermatogonial mitoses and spermatocyte meiosis progressively stop. We have never observed any inhibition of the draining of spermatozoa in the spermducts as was described for two species of locust under the action of an excess of JH during larval instars (Cantacuzène, 1968).

Effect of Altosid® on R. harrisii *oogenesis*

Ovaries of young females, exposed to JH mimic and sacrificed at a cephalothoracic length which does not go beyond 5.5 mm, present a morphology similar to ovaries of crabs of the same size reared in control media. As already described, ovaries of normal crabs (Payen, 1974a) exhibit an internal region containing gonia and oocytes with conspicuous chromosomes and, located at the periphery of the gonad, strands of oocytes in previtellogenic growth enclosed by follicle cells. No vitellogenic oocyte could be detected in ovaries of either exposed or nonexposed females. Methoprene, therefore, does not seem to affect the beginning of oogenesis, which occurs as normally as in controls.

But the situation is quite different among treated females of a cephalothoracic length over 6 mm. As opposed to those reared in control media, which essentially show darkly-pigmented oocytes ranging in diameter from 130 to 220 μm, no fully-developed oocyte can be seen in ovaries of females sacrificed after 30 days of exposure. The milky aspect of the gonads, the light acidophilic affinity of the oocyte cytoplasm and the average diameter of 85 μm reveal the incapacity to achieve vietellogenesis. After 15 days of exposure, besides gonia and oocytes with conspicuous chromosomes, some pigmented oocytes are still present; while in numerous others, further development appears blocked at terminal previtellogenesis (40 μm of average diameter) or at an early vitellogenic phase. This ultimate effect on oogenesis appears clearly in crabs sacrificed after 30 days and shows the strong effect of a JH-like compound on yolk deposition.

The presence of few pigmented oocytes in early sacrificed females can be explained by the fact that oocytes already engaged in vitellogenesis at the beginning of the experiment are able to go through the whole phenomenon without being disturbed by methoprene application. After a longer experimental period, the complete disappearance of pigmented oocytes seems due to their release in the oviducts, although no real spawning could be detected. However, also noticed in the ovaries was a rather high degree of resorption both of these oocytes, and of younger ones blocked in early vitellogenesis, by the proliferating follicle cells.

DISCUSSION

Study of female and male gametogenesis of the crab *R. harrisii* reared in the laboratory in the presence of methoprene indicates that this JH mimic acts as a chemosterilant which induces a progressive inhibition of vitellogenesis and stimulation of spermatogenesis after a short time of exposure (12–15 days), and a pronounced inhibition of both spermatogenesis and vitellogenesis after a longer delay.

Oosorption obtained after methoprene treatment is similar to the effect occurring in normal and destalked females which have experimentally received two androgenic glands (Payen, 1969, 1975). Likewise, a transitory increase of testes development, due to an androgenic hyperactivity (Payen, Costlow, and Charniaux-Cotton, 1971) preceding degeneracy, has been observed in juvenile crabs deprived of eyestalks (Payen, unpublished). Thus, after a longer experimental delay, an excess of androgenic hormone can affect the maintenance of spermatogenesis as methoprene does.

Few results concern the effects of compounds with JH activity on insect male physiology. Besides sexual behavior, it is known that endogenous JH is required for the regulation of spermatophore production in *Hyalophora cecropia* accessory glands (Williams, 1956), as well as tubular gland-ejaculatory duct and accessory gland enlargement in *Nymphalis antiopa* (Herman and Bennett, 1975). In most insect species, spermatogenesis takes place at nymphal stage and continues during the postlarval life in the presumed absence of the molting hormone (ecdysone) and presence of JH (Engelmann, 1970, p. 191; Dumser and Davey, 1974). Knowledge of the role of the latter hormone, or its mimics, in development and function of the testes is, so far, very limited. Economopoulos and Gordon (1971) did not find any modification of spermatocyte differentiation in nymphs of *Oncopeltus fasciatus* by treatment with JH analogs. Landa and Metwally (1974), using two JH analogs on pupae of the Coleoptera, mentioned the persistence of a normal spermatogenesis with one analog (Ethyl 11-chloro-3, 7, 11 trimethyl 1-2 dodecenoate), whereas a certain perturbation of the initial and middle phases of the male gametogenesis happens with the other applied analog (6, 7-epoxygeranyl 3, 4-methylendioxyphenyl). Dumser and Davey (1974) reported that farnesyl methyl ether (JH analog) produces a decline of the rate of spermatogonial mitosis and consequently a curtailment of meiotic activity on *Rhodnius prolixus*. More recently, Szöllösi (1975) observed an inhibition of the imaginal differentiation of the spermducts of two acridids but no direct effect on spermatogenesis after injection of either a synthetic Cecropia hormone or an analog (hydroprene or ZR-512) during late larval life. According to Reissig and Kamm (1975), the synthetic analog methyl 10, 11-epoxy-7-ethyl-3, 11 dimethyl-2, 6-tridecadienoate does not act on growth and development of the testes of *Draeculacephala crassicornis* in diapause, but hastens initial sperm maturation. While allatectomy or implantation of supernumerary *corpora allata* have provided evidence which implicates JH in the inhibition of testicular development (review by Dumser and Davey, 1974, page 1020), the few conflicting results obtained after treatment with JH mimics actually prevent one from drawing a similar conclusion.

In contrast to males, many studies have shown a gonadotropic effect of JH or juvenoids in adult females of several insect species (reviews by Engelmann, 1970; Sláma, Romaňuk and Sŏrm, 1974; Herman and Bennett, 1975). With a few exceptions, such as *Bombyx mori* (Bounhiol, 1942), initiation of vitellogenesis appears to be the result of the secretory activity of both the *pars intercerebralis*, located in the protocerebron, and the *corpora allata*. JH secretion, or juvenoid application, stimulates vitellogenin (female-specific protein yolk precursor) synthesis, its release from the fat body, and its incorporation into the oocytes through the follicle cells (Bell and Barth, 1971; Wyss-Huber and Lüscher, 1972; Davey and Huebner, 1974; Abu-Hakima and Davey, 1975). Response to juvenoid administration during adult life leads to anomalies or inhibition of embryonic development (reviews by Patterson, 1974; Pihan, 1975).

In decapods, it is known that spermatogenesis only takes place during the postlarval life (Payen, 1974a). Considering the fact that most of these malacostracans can molt during their whole life, male gametogenesis may occur when molting hormone is present. Although we have yet no information on the possible presence of other endocrine substances such as JH, it is remarkable to compare the similarity of our results with those relating the modifications of the respiratory and energetic metabolism of two noxious *Lepidoptera* treated with a synthetic insecticide (DU 19111) applied from the fourth larval instar (Moreau, Castex and Lamy, 1975). Initial stimulation of the metabolism followed by an important depression recalls, indeed, the phenomena which take place in the testes of *R. harrisii* treated with methoprene.

Concerning the mechanism of oocyte lysis, it is interesting to note that an analogy can be established with the amphipod *Orchestia gammarellus* after injection of synthetic JH-I (Charniaux-Cotton, 1974). Thus, methoprene and JH-I appear to act in the same inhibitory way on vitellogenesis of both amphipods and decapods; whereas, as indicated at the beginning of this work, these types of compounds are usually responsible for promoting a precocious growth of oocytes by deposition of yolk in most insect species (compare with Sláma, Romaňuk and Sŏrm, 1974, pp. 36–39 and 236–243). However, resorption of vitellogenic oocytes has been observed in reproducing females of *Thysanura*, the firebrat *Thermobia domestica* (Rohdendorf and Sehnal, 1973; Rohdendorf, 1975) following application of various juvenoids. Malacostracan crustaceans and aptergygote insects show a close relation in that they maintain the capacity to ecdyse throughout postlarval life. It, then, becomes understandable why a pterygote insect imago differs from a mature apterygote or a crustacean: in this latter arthropod, the molting glands (prothoracic or so-called ventral glands in insects and Y-organs in crustaceans) function during the whole life, whereas such organs would degenerate in most pterygotes as soon as metamorphosis is completed. From this different physiological situation, crustacean postlarval stages appear more comparable with the ptergygote larvae than their imago.

If the alterations of male and female gametogenesis are true effects, *i.e.*, really due to the hormone-like compound and not to a toxic reaction, there is no doubt that the observed results suggest certain analogies between juvenile and androgenic hormones, since both of them affect male and female gametogenesis in the same way. In fact, it appears that the ingested and topically applied JH analog is either working with an endogenous crustacean hormone closely related to insect JH or outcompeting it for the same active site. The exogenous compound may also be chemically modified to an androgenic-like hormone once it reaches the hemolymph. As already suggested above, the synthetic hormone used in the present work is neither identical or a likely precursor of the natural insect hormone but possesses the juvenilizing as well as the gonadotropic effect. Moreover, the tested concentration (1.30 ppm) probably far exceeds normal physiological doses. It is interesting to point out that no larvae of the same species survived beyond the first zoeal stage at 1.00 ppm ZR-515 under optimum or under stress conditions of salinity and temperature (Costlow, 1976; Christiansen, Costlow and Monroe, 1977).

The availability of various synthetic and natural insect hormones may provide a better understanding of arthropod physiology. For example, more experiments involving destruction or ablation of Y-organs in crabs exposed to a JH analog could complete our findings and bring new data concerning the factors required for vitellogenesis and spermatogenesis in malacostracans. The lack of the source of ecdysone would then allow an easier comparison of the results in pterygote insects and crustaceans.

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SUMMARY

1. Effects of ingestion and topical application of 1.30 ppm methoprene, an insect juvenile hormone (JH) mimic, include gametogenesis disorders in both male and female mud-crab *Rhithropanopeus harrisii.*

2. Progressive inhibition of vitellogenesis and stimulation of spermatogenesis take place after a short exposure time (12–15 days), whereas a longer period (between 30 to 45 days) promotes inhibition of both spermatogenesis and vitellogenesis. Such modifications demonstrate the chemosterilizing action of methoprene on crustaceans.

3. Stoppage of vitellogenesis is characterized by a blockage of oocytes at terminal previtellogenesis and proliferation of the follicle cells involved in vitellogenic oocyte lysis. Spermatogenic degeneration begins with the amalgam of spermatocyte clusters in meiotic prophase and pycnosis of gonia.

4. Analogies between these results and those obtained from experiments involving androgenic gland implantations or the consequences of their hyperfunctioning are discussed.

LITERATURE CITED

Abu-Hakima, R., and K. G. Davey, 1975. Two actions of juvenile hormone on the follicle cells of *Rhodnius prolixus* Stal. *Can. J. Zool.*, **53**: 1187–1188.

Bell, W. J., and R. H. Barth Jr., 1971. Initiation of yolk deposition by juvenile hormone. *Nature, New Biol.*, **230**: 220–222.

Bounhiol, J. J., 1942. L'ablation des corps allates au dernier âge larvaire n'affecte pas la reproduction chez *Bombyx mori*. *C. R. Hebd. Seances Acad. Sci. Paris Ser. D Sci. Nat.* **215**: 234–236.

Bowers, W. S., 1971. Juvenile hormones. Pages 307–332 in M. Jacobson and D. G. Crosby, Eds., *Naturally occurring insecticides*. M. Dekker, New York.

Byard, E. H., and R. R. Shivers, 1975. The mandibular organ of Lobster, *Homarus americanus*. *Cell. Tissue Res.*, **162**: 13–22.

Cantacuzène, A. M., 1968. Différenciation des caractères sexuels mâles de deux Acridiens après implantation des corps allates pendant les derniers stades larvaires. *C. R. Hebd. Seances Acad. Sci. Paris Ser. D Sci. Nat.*, **267**: 749–752.

Charniaux-Cotton, H., 1974. Données nouvelles concernant la vitellogenèse des Crustacés Malacostracés obtenues chez l'Amphipode *Orchestia gammarellus* (Pallas); folliculogenèse à partir d'un tissu permanent; action du busulfan; action inhibitrice de l'hormone juvénile. *C. R. Hebd. Seances Acad. Sci. Paris Ser. D Sci. Nat.*, **279**: 563–566.

Christiansen, M. E., J. D. Costlow, Jr., and R. J. Monroe, 1977. Effect of the juvenile hormone mimic ZR-515 (Altosid®) on the larval development of the mud-crab *Rhithropanopeus harrisii* in various salinities and cyclic temperatures. *Mar. Biol.*, **39**: in press.

Costlow, J. D., Jr., 1976. The effect of the juvenile hormone mimics on development of the mud-crab, *Rhithropanopeus harrisii* (Gould). Pages 439–459 in A. Calabrese, F. P. Thurberg, and F. J. Vernberg, Eds., *Proc. symp. on pollution and physiology of marine organisms*. Academic Press, New York.

Crochard, C., 1975. Définition du test "Grillon" d'activité ovidice des analogues de l'hormone juvénile des insectes. *C. R. Hebd. Seances Acad. Sci. Paris Ser. D. Sci. Nat.*, **281**: 61–64.

Davey, K. G., and E. Huebner, 1974. The response of the follicle cells of *Rhodnius prolixus* to juvenile hormone and antigonadotropin *in vitro*. *Can. J. Zool.*, **52**: 1407–1412.

Dumser, J. B., and K. G. Davey, 1974. Endocrinological and other factors influencing testis development in *Rhodnius prolixus*. *Can. J. Zool.*, **52**: 1011–1022.

Dunham, L. L., D. A. Schooley, and J. B. Siddall, 1975. A survey of the chromatographic analysis of natural insect juvenile hormones and the insect growth regulator, Altosid®. *J. Chromatogr. Sci.*, **13**: 334–336.

Economopoulos, A. P., and H. T. Gordon, 1971. Growth and differentiation of testes in the large milkweed bug, *Oncopeltus fasciatus* (Dallas). *J. Exp. Zool.*, **177**: 391–405.

Engelmann, F., 1970. *The physiology of insect reproduction*. International Series of monographs in pure and applied biology, Vol. 44. Zoology division, Pergamon Press, Oxford, 307 pp.

Gomez, E. D., D. J. Faulkner, W. A. Newman, and C. Ireland, 1973. Juvenile hormone mimics: effect on cirriped crustacean metamorphosis. *Science*, **179**: 813–814.

Henrick, C. A., G. B. Staal, and J. B. Siddall, 1973. Alkyl 3, 7, 11-trimethyl-2, 4 dodecodienoates, a new class of potent insect growth regulators with juvenile hormone activity. *J. Agr. Food Chem.*, **21**: 354–359.

Herman, W. S., and D. C. Bennet, 1975. Regulation of oogenesis, female specific protein production, and male and female reproductive gland development by juvenile hormone in the butterfly, *Nymphalis antiopa*. *J. Comp. Physiol.*, **99**: 331–338.

Ittycheriah, P. I., E. P. Marks, and M. S. Quraishi, 1974. Effects of hormones, brain, and endocrine glands on the ovary of *Culex tarsalis in vivo* and *in vitro*. *Ann. Entomol. Soc. Am.*, **67**: 595–600.

Landa, V., and M. M. Metwally, 1974. Effects of conventional chemosterilants and juvenile hormone analogues on spermatogenesis in *Trogoderma granarium* (Coleoptera, Dermestidae). *Acta Entomol. Bohemoslov.*, **71**: 145–152.

Moreau, R., C. Castex, and M. Lamy, 1975. Examen préliminarie de quelques aspects des effets métaboliques d'un nouvel insecticide de synthèse chez deux insectes nuisibles:

Pieris brassicae L. et *Thaumetopoea pityocampa* S. (Lépidoptères). *Ann. Zool. Ecol. Anim.*, **7**: 161–170.

Novák, V. J. A., 1971. Juvenile hormone analogues and their theoretical and practical significance. *Acta Phytopathol. Acad. Sci. Hung.*, **6**: 203–210.

Patterson, J. W., 1974. A comparison of the morphogenetic and sterilizing activities of Juvenile Hormone mimics on *Aedes aegypti*. *J. Insect. Physiol.*, **20**: 2095–2106.

Payen, G., 1969. Expériences de greffes de glandes androgènes chez la femelle pubère du Crabe *Rhithropanopeus harrisii* (Gould) (Crustacé, Décapode). *C. R. Hebd. Seances Acad. Sci. Paris Ser. D Sci. Nat.*, **268**: 393–396.

Payen, G., 1974a. Morphogenèse sexuelle de quelques Brachyoures (Cyclométopes) au cours du développement embryonnaire, larvaire et postlarvaire. *Bull. Mus. Natl. Hist. Nat. Paris Ser. 3, 209 Zool.*, **139**: 201–262.

Payen, G., 1974b. Recherches sur la réalisation et le contrôle de la différenciation sexuelle chez les Crustacés Décapodes Reptantia. *Thèse d'Etat*, Paris, AO, CNRS-9930, 224 pp.

Payen, G., 1975. Effets masculinisants des glandes androgènes implantées chez la femelle pubère pédonculectomisée de *Rhithropanopeus harrisii* (Gould) (Crustacé Décapode Brachyoure). *C. R. Hebd. Seances Acad. Sci. Paris Ser. D Sci. Nat.*, **280**: 1111–1114.

Payen, G., J. D. Costlow, and H. Charniaux-Cotton, 1971. Etude comparative de l'ultrastructure des glandes androgènes de Crabes normaux et pédonculectomisés pendant la vie larvaire ou après la puberté chez les espèces *Rhithropanopeus harrisii* (Gould) et *Callinectes sapidus* Rathbun. *Gen. Comp. Endocrinol.*, **17**: 526–542.

Pihan, J. C., 1975. Utilisation des effets tératogènes des hormones juvéniles et de leurs mimétiques dans la lutte contre les insectes. *Année Biol.*, **14**: 29–44.

Ramenofsky, M., D. J. Faulkner, and C. Ireland, 1974. Effect of juvenile hormone on cirriped metamorphosis. *Biochem. Biophys. Res. Commun.*, **60**: 172–178.

Reddy, G., and A. Krishnakumaran, 1973. Changes in the morphogenetic response of *Tenebrio molitor* to juvenile hormone in relation to age. *J. Insect Physiol.*, **19**: 773–780.

Reissig, W. H., and J. A. Kamm, 1975. Effect of synthetic juvenile hormone on reproduction of adult male *Draeculacephala crassicornis*. *Ann. Entomol. Soc. Am.*, **68**: 353–354.

Rohdendorf, E. B., 1975. Histological changes in the ovaries of *Thermobia domestica* (Thysanura) following application of juvenoids. *Acta Entomol. Bohemoslov.*, **72**: 209–220.

Rohdendorf, E. B., and F. Sehnal, 1973. Inhibition of reproduction and embryogenesis in the firebrat, *Thermobia domestica* by juvenile hormone analogues. *J. Insect Physiol.*, **19**: 37–56.

Roussel, J. P., and J. M. Perron, 1974. Action de substances mimétiques de l'hormone juvénile sur *Schistocerca gregaria* Forsk. *Arch. Zool. Exp. Gén.*, **115**: 251–262.

Sláma, K., 1971. Insect juvenile hormone analogues. *Annu. Rev. Biochem.*, **40**: 1079–1102.

Sláma, K., M. Romaňuk, and Sŏrm, 1974. *Insect hormones and bioanalogues*. Springer-Verlag, Wien, New York, 477 pp.

Stockel, J., 1975. Les analogues de l'hormone juvénile, ou régulateurs de croissance chez l'insecte. Possibilités d'application dans la lutte contre les espèces nuisibles. *Ann. Zool. Ecol. Anim.*, **7**: 91–118.

Szöllösi, A., 1975. Imaginal differentiation of the spermiduct in acridids: effects of juvenile hormone. *Acrida*, **4**: 205–216.

Williams, C. M., 1956. The juvenile hormone of insects. *Nature*, **178**: 212–213.

Wyss-Huber, M., and M. Lüscher, 1972. *In vitro* synthesis and release of proteins by fat body and ovarian tissue of *Leucophaea maderae* during the sexual cycle. *J. Insect. Physiol.*, **18**: 689–710.

Yu, S. J., and L. C. Terriere, 1975. Microsomal metabolism of juvenile hormone analogs in the housefly, *Musca domestica* L. *Pest. Biochem. Physiol.*, **5**: 418–430.

Reference: *Biol. Bull.*, **152**: 209–220. (April, 1977)

FURTHER OBSERVATIONS ON THE STRUCTURE AND FUNCTION OF THE OPERCULUM IN *SPIRORBIS MOERCHI* (SERPULIDAE: SPIRORBINAE)

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An important aspect of the reproductive biology of the ubiquitous Spirorbinae is the fact that all known species brood their embryos until larval release. Brood protection occurs either within the parental tube or within a modified operculum, and the mode of brood protection has been proposed as a basis for taxonomic reclassification (Bailey, 1969). Of the two types of brood protection, incubation within the operculum is the most specialized and has been considered a recent development in the evolution of the subfamily (Elsler, 1907; Borg, 1917; Gravier, 1923). Just how newly spawned oocytes enter the opercular brood chamber remains controversial. Oocytes have been described as traveling through the thoracic coelom and subsequently being squeezed into the brood chamber *via* the opercular peduncle (Vuillemin, quoted by Knight-Jones and Knight-Jones, 1974). Such a mechanism would be impossible in *Spirorbis moerchi* because the opercular peduncle is solid and the only entrance into the brood chamber is by means of a pore in the opercular epithelium (Potswald, 1968). The opercular pore in *S. moerchi* has been observed to open wide enough to allow the release of larvae. It can be argued that opening of the opercular pore during larval release is simply a passive phenomenon resulting from force applied by the moving larvae; however, in most cases, the operculum receives another brood shortly after larval release and the pore is then observed to be tightly closed, suggesting that closing of the pore is an active process. It can be further argued that opening, like closing, of the pore may be under active control because the pore appears closed in a virgin operculum prior to deposition of the first brood (Potswald, 1968).

There have been recent studies on opercular histology in several species of *Spirorbis* (Thorp, 1975; Thorp and Segrove, 1975), including one electron microscopic investigation (Bubel, 1973). The latter study, however, fails to give any detailed information concerning the epithelium surrounding the pore. It is the purpose of the present report to provide ultrastructural observations on the opercular epithelium of *S. moerchi*, with particular attention being paid to the epithelium of the pore region in brooding and nonbrooding individuals, in an attempt to correlate morphological detail with function. The results to be presented are an extension of those reported in an earlier study of opercular histology at the resolution of light microscopy (Potswald, 1968).

Materials and Methods

Adult specimens of *Spirorbis moerchi* were collected in Argyle creek on San Juan Island, state of Washington. Worms were removed from their calcareous tubes by means of heavy dissecting needles. Opercular brood pouches containing embryos, at various stages of development, together with pouches from which larvae recently emerged, were removed at the base of the opercular crown with #5 watchmaker's forceps. The brood pouches were fixed in an ice-cooled mixture of one part 5% glutaraldehyde, one part 5% osmium tetroxide, and two parts of 0.2 M phosphate buffer at pH 7.4 (Stanley, 1967). Numerous attempts to use glutaraldehyde, utilizing a number of different buffers and made isotonic with sea water, as a primary fixative have not been successful with this material. Following fixation, the opercular brood pouches were dehydrated in a graded series of alcohols from 30% to absolute and then embedded in Epon 812 (Luft, 1961). Brood pouches were flat embedded, cut out of block, and oriented, in three planes, on chucks. Each specimen was pared down with a razor blade to just within the region of the pore. One micron thick sections were then cut with a Porter Blum MT-1, affixed to glass slides and stained with Richardson's stain (Richardson, Jarett, and Finke, 1960). Adjacent thin sections were cut with a diamond knife, picked up on 200 mesh grids, and stained in saturated 50% alcoholic uranyl acetate for 20 minutes followed by lead citrate reagent (Reynolds, 1963) for five minutes. The specimens were examined and photographed with an RCA EMU-3E electron microscope.

RESULTS

The operculum of *Spirorbis moerchi* contains a spacious cavity, the brood chamber, and is capped by a characteristically bilobed calcareous plate (Fig. 1). As observed from the side with a dissecting microscope, a thickening, indicating the site of the opercular pore, can be seen in the opercular epithelium. A section through a brood pouch containing embryos shows that the epithelium surrounding the opercular pore is simple columnar, the cells varying in height from about 20 to 25 microns (Fig. 2). A distinct cuticle can be seen to cover the epithelium and during brooding the cuticle tends to almost completely occlude the opercular opening. Light microscopy fails to provide evidence for the presence of muscle in the area surrounding the opercular pore.

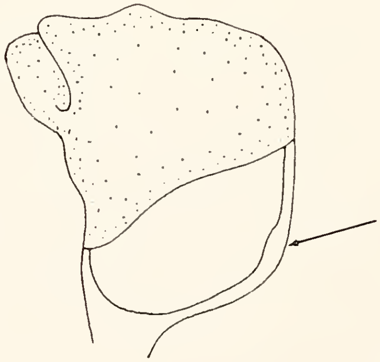
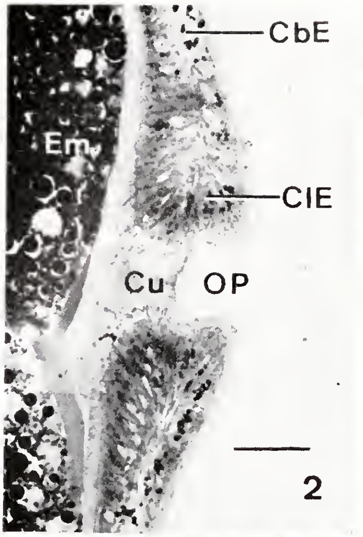


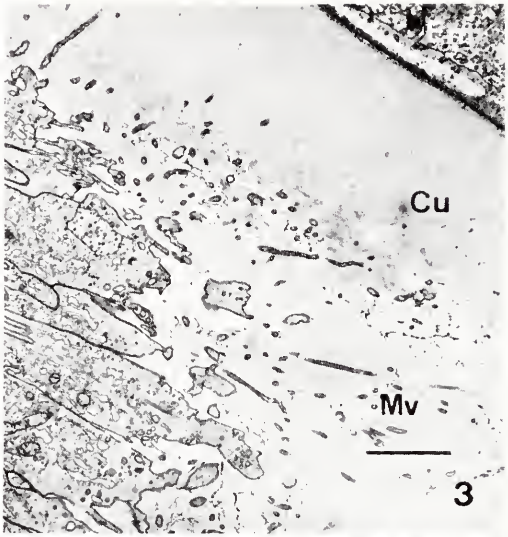
FIGURE 1. Drawing of a lateral view of a brooding operculum of *S. moerchi* showing the bilobed calcareous plate (stippled area), and the thickened region of the brood pouch epithelium indicating the site of the opercular pore (arrow). Embryos not shown.

An examination of the pore epithelium with the electron microscope during brooding reveals that the epithelial cells possess a narrow diameter from base to apex (Figs. 3, 4). Long irregular microvilli extend from the apex of the cells into the cuticle which nearly occludes the pore (Fig. 3). The cells are joined together by subapical septate junctions arranged in zonulae; however, at the apex, the cells are separated by a relatively wide intercellular space which extends for a considerable length (Figs. 3, 5, 6). In addition to septate junctions, the lateral surfaces of the cells exhibit simple interdigitations (Figs. 3, 4). A distinct basal lamina is absent and the inner and outer epithelium of the pore is separated by an intercellular space containing reticular-like fibers (Fig. 4). Elongate nuclei are basally situated and each contains a nucleolus and scattered chromatin. Dense granules, probably representing pigment responsible for the orange coloration of the operculum in life, are found throughout the cytoplasm (Fig. 4). Portions of the dense granules leach out during fixation, suggesting that the granules contain lipid, perhaps associated with the suspected carotenoid. Each cell contains a well-developed Golgi complex apical to the nucleus together with multivesicular bodies and numerous individual vesicles (Fig. 5). Besides the afore-mentioned organelles and inclusions, the cytoplasm contains mitochondria of conventional morphology, free ribosomes, and relatively little endoplasmic reticulum.

Aside from shape, the most interesting aspect of the cells surrounding the pore during brooding is the presence of numerous microfilaments. The microfilaments, 50–70 Å in diameter, are found individually throughout the cytoplasm and form a dense, felt-like, circumferential network adjacent to the apical plasmalemma. Microfilaments are also seen forming bundles which run parallel to the axis of the cell and extend from the base apically into the microvilli (Figs. 4, 5, 6). The bundles of microfilaments do not appear to attach basally.

The columnar epithelium surrounding the opercular pore is continuous with the cuboidal epithelium which comprises the rest of the noncalcified portion of the opercular brood pouch. The outer cuboidal epithelial layer is about 6 microns thick; whereas, the inner epithelial layer is somewhat thinner (Fig. 7). As in the region of the pore, the inner and outer epithelial layers are separated by an intercellular space containing reticular-like fibers. Unlike the region of the pore, a distinct basal lamina can be observed underlying inner and outer epithelia. Both inner and outer epithelial layers have apical microvilli which penetrate the cuticle; however, the microvilli are much shorter and more regular in appearance than in the pore region. Dense granules, presumably pigment, and intracellular vacuoles are present in both inner and outer epithelia, but are more prevalent in the latter. The vacuoles tend to be much smaller in brooding than in nonbrooding opercula, and, in fact, are difficult to observe in brooding opercula at the light microscopic level (Potswald, 1968). Bundles of microfilaments are present in both epithelial layers, but, contrary to the bundles observed in the columnar epithelium surrounding the pore, these definitely attach to basal hemidesmosomes (Fig. 7, inset). Other than the above-mentioned differences, the cytoplasmic organelles are similar to those observed in the pore epithelium. One further point of interest is the fact that the cells comprising the inner and outer cuboidal epithelia are closely apposed apically and do not exhibit the intercellular spaces observed separating the apical portions of the columnar cells surrounding the pore of a brooding operculum.





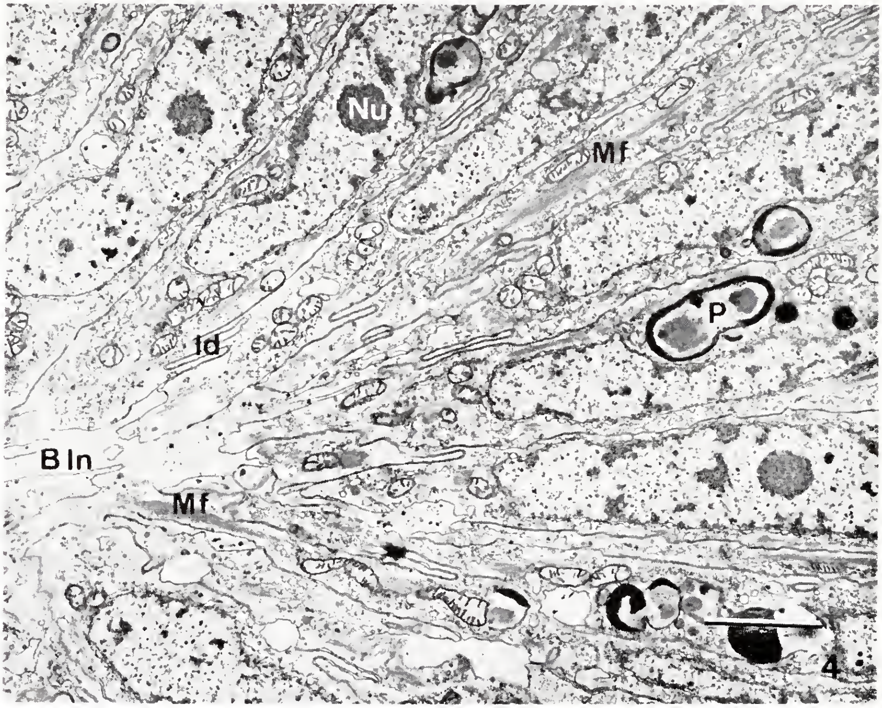


FIGURE 2. Light micrograph of a sagittal section through a brooding operculum showing the columnar epithelium (ClE) bordering the opercular pore (OP), cuboidal epithelium (CbE), cuticle (Cu), and embryos (Em) within the brood chamber. Scale equals 20 μ.

FIGURE 3. A thin section through the apical region of the columnar epithelium bordering the pore of a brooding operculum. Note the narrow diameter of the epithelial cells, the long microvilli (Mv) penetrating the filamentous cuticle (Cu), and a portion of an embryo (Em) within the brood chamber. Scale equals 2 μ.

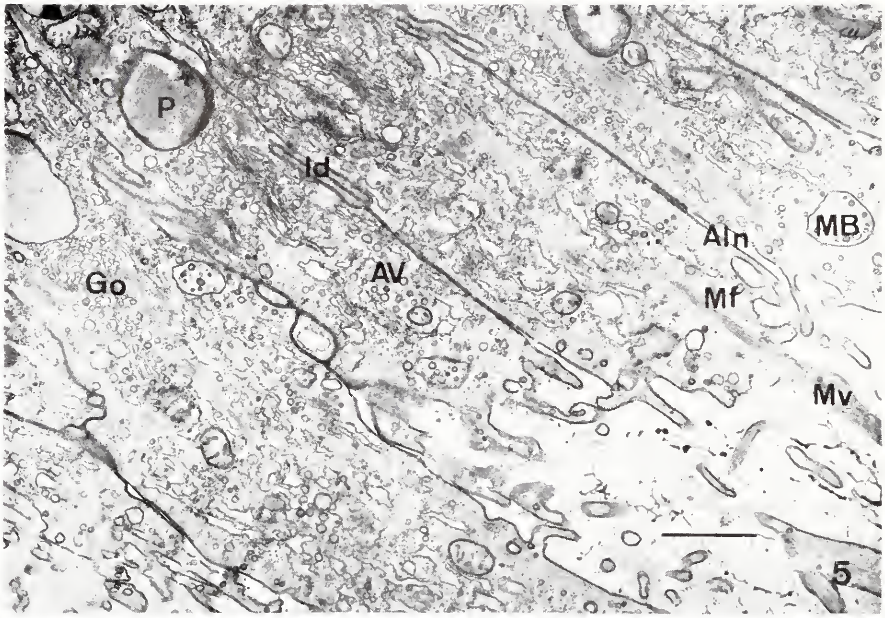
In an attempt to observe changes in the columnar epithelium when the opercular pore is wide open, brood pouches were fixed after the emergence of larvae. Ideally, one would like to observe changes in the epithelium immediately after the release of larvae. Unfortunately, larval release has been observed fortuitously only on a few occasions (Potswald, 1968); consequently, the following observations are based on opercula fixed within 12 hours of larval release and prior to the deposition of a new brood. In each of the three cases to be reported on, the opercular pore, as determined by examination with a dissecting microscope, was open but not maximally, *i.e.,* not wide enough to allow the simultaneous release of two larvae at a time. Further difficulties were encountered upon fixation when the opercular pores tended to close still further upon contact with the fixative. Nevertheless, some interesting differences have been observed in the pore epithelium of nonbrooding opercula.

Upon widening of the brood pore during larval release, one would predict that the previously columnar epithelial cells closing the pore would change shape and take on the characteristics of a cuboidal or even squamous epithelium. This prediction seems to be realized even in opercula whose pores are only perhaps half open (Fig. 8). The cells are reduced in height and consequently exhibit a greater diameter and concomitant reduction in length of microvilli as compared with the columnar cells of brooding opercula. With a shortening of the cells there is a corresponding decrease in the length and width of the intercellular spaces seen separating the columnar cells apically in the brooding condition. Since the empty brood pouches observed here did not have maximally opened pores, one might expect to see cells intermediate in shape between the tall columnar cells of the brooding operculum and the shortened condition of the epithelium surrounding the partially opened pore of the nonbrooding operculum. Such cells have been observed, but they are not nearly as columnar as those surrounding the pore of a brooding operculum (Fig. 8). Bundles of microfilaments running parallel to the cell axis, as observed in the columnar cells of the brooding operculum, are absent in the cells surrounding the opening of the nonbrooding operculum. Instead, the microfilaments form a rather dense mat just below the flattened apical plasma membrane (Fig. 8). In lateral view, the feltwork of microfilaments can be seen to exclude membranous organelles from the apical region. *En face* views of sections taken parallel to and just below the free surface of the epithelium show microfilaments coursing near the lateral cell membranes (Fig. 9). A dense region closely applied to the cytoplasmic surface of the cell membrane suggests an intimate association between microfilaments and the lateral plasmalemma. Although the extensive filamentous network is absent, it will be recalled that the circular dense band of membrane associated microfilaments is present apically in the columnar cells of the brooding operculum.

DISCUSSION

Although the epithelium comprising the opercular wall in *Spirorbis moerchi* is a double layer, it is, nevertheless, a simple epithelium. Both the inner and the

FIGURE 4. Basal region of the columnar epithelium of a brooding operculum showing elongate nuclei, each of which contains a single nucleolus (Nu), dense granules believed to be pigment (P), lateral interdigitations (Id), basal intercellular space (BIn), and longitudinal bundles of microfilaments (Mf). Scale equals 2 μ.



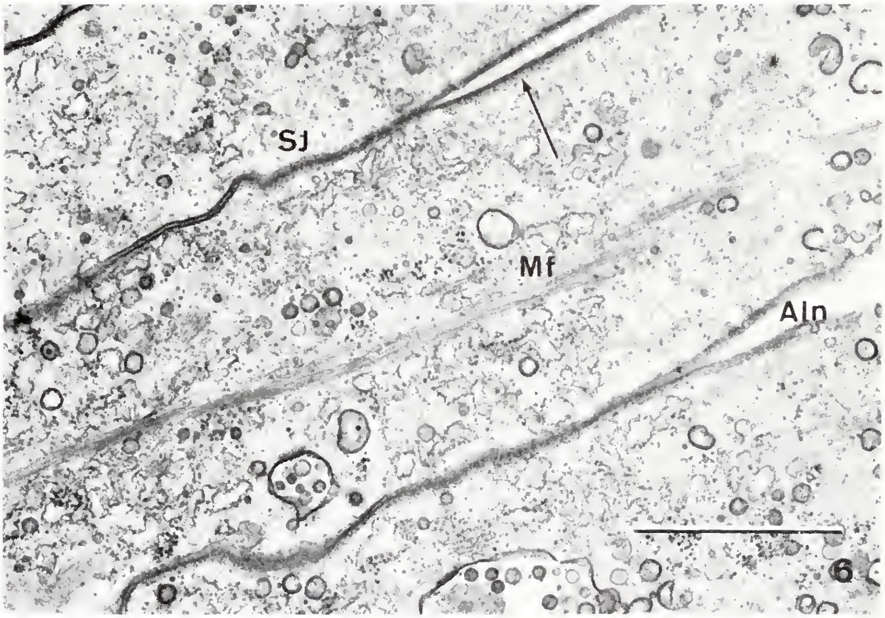


FIGURE 5. Apical region of the pore epithelium of a brooding operculum showing that each cell has an apical Golgi complex (Go), multivesicular bodies (MB), pigment granules (P), lateral interdigitations (Id), apical vesicles (AV), microvilli (Mv), and longitudinal bundles of microfilaments (Mf). Note also that the epithelial cells are separated by apical intercellular spaces (AIn). Scale equals 1 μ.

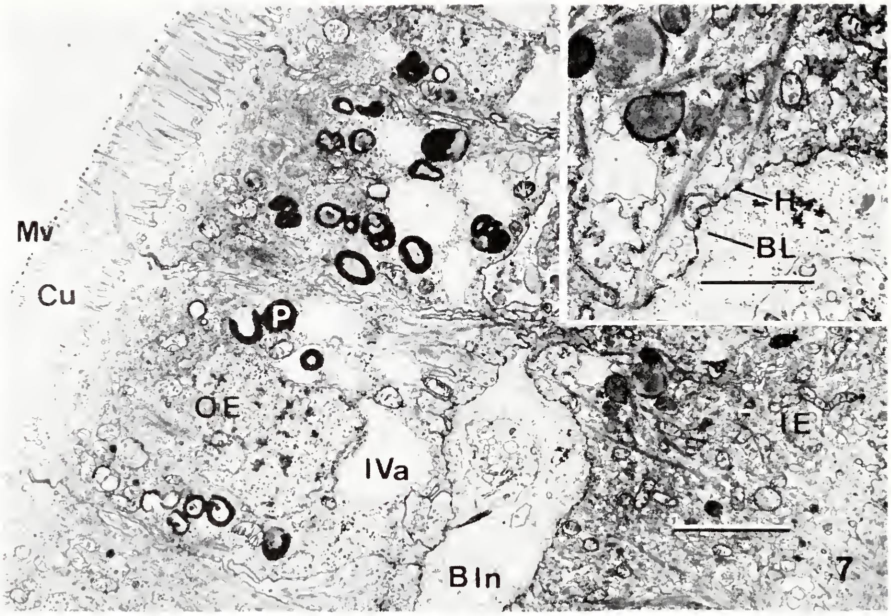
FIGURE 6. Similar to Figure 5 but at higher magnification, showing septate junctions (SJ), apical intercellular spaces (AIn), longitudinal bundles of microfilaments (Mf), and circumferential network of microfilaments adjacent to the apical plasmalemma (arrow). Scale equals 1 μ.

outer epithelium has its own basal lamina; sandwiched between, and separating the latter, is a reticular lamina. As the two layers converge upon the opercular pore, a basal lamina is difficult to demonstrate and the cuboidal epithelial layers become confluent with the columnar epithelium bordering the pore. How the double nature of the opercular epithelium arises has been explained by Thorp (1975). According to Thorp, during development, the opercular ampulla is composed of a single epithelial layer. As development proceeds, the ampullar epithelium involutes, leaving the opercular pore at the site of involution. This would account not only for the double nature of the opercular epithelium, but also for the presence of an inner cuticle. It also helps explain why the calcareous plate in *S. moerchi* forms inside the brood chamber between the inner epithelium and inner cuticle (Potswald, 1968). A fine structural study of opercular development would be of interest to determine whether, as might be expected, microfilaments play a role in the process of ampullar involution.

The epithelium making up the epidermis of the annelidan body wall has been described as containing basal cells, specialized gland cells, and exhibiting a pseudostratified appearance (Coggeshall, 1966; Potswald, 1971; Burke, 1974). Although the latter features are not found in the operculum, the septate junction in the form of a belt encircling the cell, apical microvilli, and cuticle are characteristics common to both body wall epidermis and the opercular epithelium. The filamentous cuticle of the brood pouch is similar to the epidermal cuticle described in a few species of polychaetes by Storch and Welsch (1970), and it in no way differs from the rest of the cuticle covering the body of *S. moerchi* (Potswald, unpublished observation). Bubel's (1973) description of the opercular cuticle in *S. borealis* is similar to that given here for *S. moerchi;* however, he reports that the opercular cuticle in *S. granulatus* and *S. pusilloides* is much more complex and contains distinctly ordered fibers. It would appear, as pointed out by Storch and Welsch (1970), that there is no correlation between the degree of cuticular complexity and the type of environment inhabited by polychaetes. There is still much to be learned concerning the formation and maintenance of the cuticle in polychaetes; however, it is likely that the well-developed apical Golgi complex found in both cuboidal and columnar cells of the opercular epithelium may play an important role, a suggestion also offered by Bubel (1973).

Dense granules present in the epithelial cells of the brood pouch, which are interpreted to be pigment granules imparting the orange color to the operculum in life, are similar to the dense granules observed in the epidermal cells of *Lumbricus* (Coggeshall, 1966) and *Eisenia* (Burke, 1974). Coggeshall suggests that the granules in *Lumbricus* may be either melanin or lipofuscin in nature. Burke points out that they may be lysosomal and cites evidence for the lysosomal nature of liposfuscin granules in other tissues. In addition to possible lysosomes, Bubel (1973) describes electron dense inclusion bodies in the opercular epithelium of *S. borealis* which he believes may represent a type of secretory product. Information presented in this report on the dense granules in *S. moerchi* does little to settle the question, but the granules appear to be the best candidates to explain coloration of the living epithelium.

A rather interesting aspect of the fine structure of the opercular epithelium is the presence of intracellular vacuoles. In *S. moerchi,* the vacuoles predominate in the cuboidal epithelia of the brood chamber, but they are apparently not unique



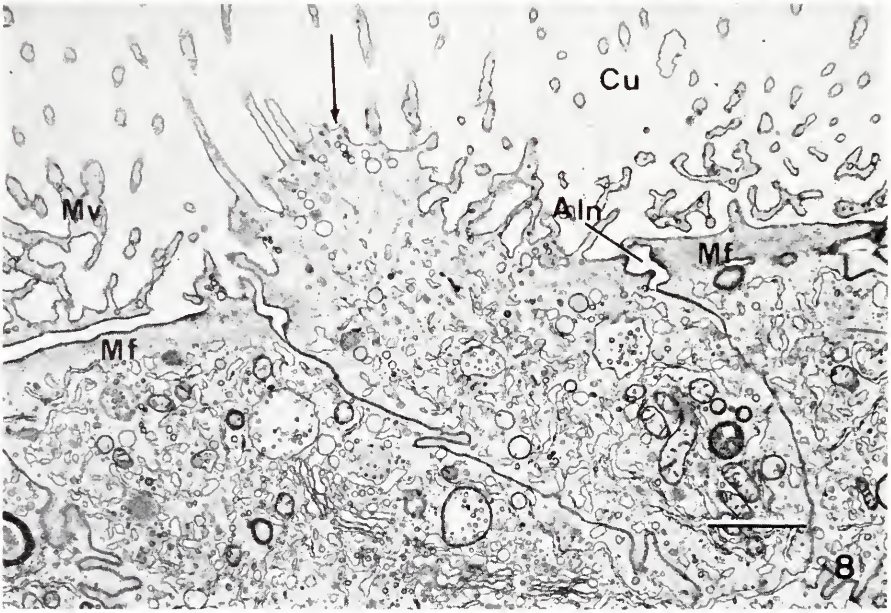


FIGURE 7. A section through the inner (IE) and outer (OE) cuboidal epithelial layers adjacent to the columnar pore epithelium of a brooding operculum showing outer cuticle (Cu), microvilli (Mv), pigment granules (P), and basal intercellular space (BIn). Note that the intracellular vacuoles (IVa) are larger and more abundant in the outer as compared with the inner epithelium. Scale equals 2 μ. The inset (upper right) shows the outer basal lamina (BL) and bundles of filaments attaching to hemidesmosomes (H). Scale equals 1 μ.

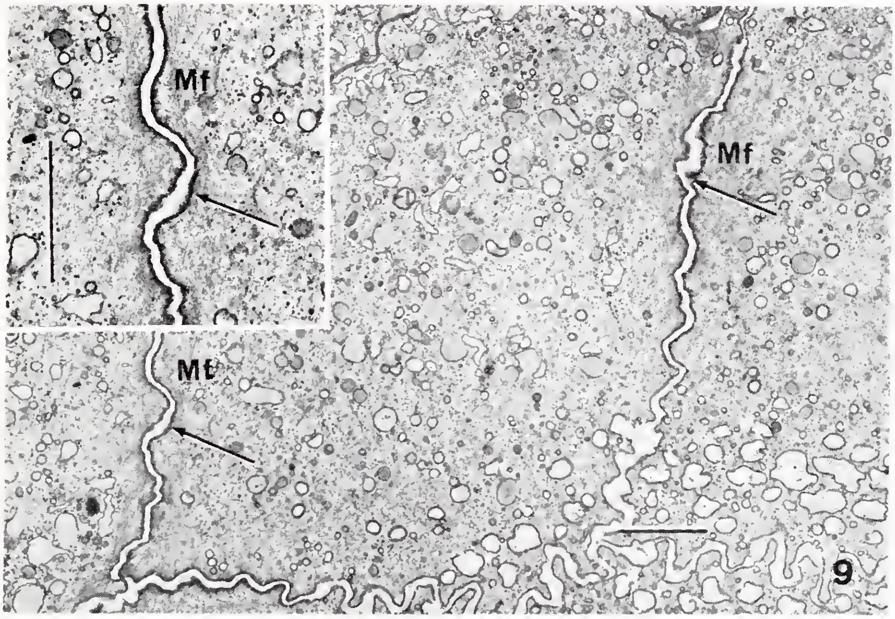


FIGURE 9. *En face* subapical view of the pore epithelium of a nonbrooding operculum showing microfilaments (Mf) coursing near the lateral cell membrane and dense region adjacent to the membrane (arrows). Scale equals 1 μ. Inset (upper left) is an enlargement of the lower left portion of Figure 9. Scale equals 1 μ.

to the epithelial cells of the brood chamber. After examining the epidermis in 18 polychaete species, Storch and Welsch (1970) describe the presence of numerous intracellular vacuoles in four species (*Autolytus pictus, Ophiodromus flexuosus, Branchiomma bombyx, Myxicola infundibulum*) and report that the largest part of the cytoplasm of the epidermal cells of *Chaetopterus variopedatus* is occupied by one large vacuole. The latter investigators could find no evidence that the vacuolated cells were restricted to certain systematic groups or species inhabiting a particular environment. Be this as it may, the very presence of the intracellular vacuoles would seem to suggest that they must have some function. Bubel (1973) has suggested that the vacuoles may play a role in cuticle and/or basal lamina secretion. The latter hypothesis is quite plausible and could be tested with high resolution autoradiography. At the light microscopic level, the vacuoles in *S. moerchi* are more apparent in nonbrooding opercula than in brooding opercula (Potswald, 1968). Such an observation might be taken to indicate that the intracellular vacuoles may function to allow the epithelium to expand upon reception of a brood. If the vacuoles are to collapse and thereby cause an expansion of the opercular epithelium, the epithelium must have a mechanism by means of which the contents of the vacuoles are expelled. The pinocytotic

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FIGURE 8. Apical region of the pore epithelium of a nonbrooding operculum showing the filamentous cuticle (Cu), microvilli (Mv), decreased apical intercellular space (AIn), and dense mat of microfilaments (Mf) below the flattened apical plasma membrane. Note that one of the cells appears to be elongating (arrow). Scale equals 1 μ.

vesicles observed in the apical ends of the opercular epithelial cells could possibly serve to accomplish this feat. It is just as reasonable to suggest, however, that the pinocytotic vesicles are of Golgi origin and are maintaining the cuticle.

Evidence has previously been presented (see Introduction) which supports the contention that the opercular pore in *S. moerchi* is capable of actively closing and perhaps opening. Light microscopy has revealed the presence of longitudinal muscle fibers embedded within the connective tissue of the opercular peduncle (Potswald. 1968; Thorp and Segrove, 1975); however, the present study has conclusively shown that muscle fibers do not penetrate between the two epithelial layers making up the opercular brood chamber. Since the important study of Cloney (1966) on the contractile role of the caudal epithelium during tail resorption in ascidian larvae, numerous reports have documented the close correlation between contraction and the presence of cytoplasmic microfilaments in a variety of nonmuscle cells (Baker and Schroeder, 1967; Schroeder, 1969, 1970, 1972, 1973; Spooner, Yamada, and Wessells, 1971; Wessells, Spooner, Ash, Bradley, Luduena, Taylor, Wrenn, and Yamada, 1971; Conrad, 1973; just to cite a few). That cytoplasmic microfilaments, as observed in fixed and embedded tissue cells, are real and not artifacts of preparation has been convincingly argued by Buckley (1975). Although experimental evidence is presently lacking, it seems reasonable to suggest that the presence of microfilaments in the columnar cells bordering the opercular pore in *S. moerchi* are responsible for the cellular shape changes observed in brooding and nonbrooding opercula.

In the tall columnar cells bordering the closed pore of a brooding operculum, microfilaments are found individually throughout the cytoplasm and form a circumferential, dense, felt-like network adjacent to the apical plasmalemma. The latter may correspond to the subplasmalemmal filament network described by Buckley (1975) in cultured cells. In addition to the network of microfilaments, longitudinal bundles of microfilaments are also observed within the columnar cells bordering the pore and, unlike bundles of filaments seen in the cuboidal epithelium of the operculum apparently do not attach to hemidesmosomes. Whether these longitudinal bundles correspond to the stress fibers observed in tissue culture cells (Buckley, 1975) is not clear. In nonbrooding opercula with partially opened pores, the cells bordering the opening show decreased height and a shortening in length of apical intercellular spaces. Microfilaments form a network under the apical plasma membranes of the flattened cells, and, subapically, a ring of microfilaments closely applied to the lateral plasmalemma can be seen. Bundles of microfilaments arranged parallel to the longitudinal axis, as seen in the brooding condition, are absent. Investigators of nonmuscle cells capable of contraction (see references cited earlier) when considering the role of microfilaments in causing cell constriction, which in the case of *S. moerchi* would lead to closure of the opercular pore, all propose that rings of microfilaments are mechanically responsible for the contractile event. Observations on opercular pore function in *S. moerchi* are consistent with the afore-mentioned hypothesis. For reasons that have already been explained, a completely "open" or "relaxed" pore condition has not been observed in *S. moerchi*; consequently, the description given for the pore epithelium in the nonbrooding operculum is a description for an epithelium in the process of contracting. As apical contraction proceeds, the cells elongate and close the pore. The apical ring of membrane associated microfilaments is retained in the brooding condition and is believed to maintain the columnar shape of the epithelial cells. During larval release, widening of the pore may not be due entirely to changes in the columnar epithelium bordering the pore. Whereas the columnar epithelium approaches the cuboidal or even squamous condition, the cuboidal epithelium in the noncalcified region of the operculum may also change shape and become more columnar. The latter possibility should be explored.

Just what controls opening and closing of the opercular pore is not known. Larval release *via* the pore has been observed in *S. moerchi* (Potswald, 1968), and it is certain that the pore remains open wide enough to accept another brood. Opening may be a passive phenomenon due to the movement of the larvae within the brood chamber but after receiving a new brood, the pore closes and this would seem to be an active process. Perhaps when newly spawned oocytes are packed into the brood chamber, recently vacated by larvae, the opercular epithelium stretches, and this stretching somehow triggers contraction of the pore epithelium. More difficult to explain, however, is how oocytes are transferred into a virgin operculum in which the pore, at least by examination with a dissecting microscope, appears to be closed (Potswald, 1968). Thorp and Segrove (1975) have described the oocytes of *S. spirorbis* as being very fluid during spawning and capable of flowing through an opening with a diameter one-tenth or less that of the oocyte itself. If the oocytes of *S. moerchi* are equally malleable, and they may well be since it is believed that they squeeze through the body wall during spawning (Potswald, 1967), then perhaps it is not too difficult to understand how they gain entrance into a virgin operculum, given that the pore is at least partially patent. However, if the pore of a virgin operculum does in fact actively open to receive the first brood, one would have to postulate the existence of some sort of stimulus which would cause a relaxation of the apical ring of microfilaments and a concomitant contraction of the epithelial cells in an apical to basal direction. The bundles of microfilaments observed parallel to the long axes of the columnar cells in the brooding condition, assuming they are also present in the virgin operculum, could possibly play a role in the hypothetical event. Unfortunately, as Thorp and Segrove (1975) point out, neither they nor any other worker have ever observed the actual transfer of oocytes into an opercular brood chamber.

I wish to thank Dr. Robert L. Fernald, retired Director of the Friday Harbor Laboratories, for providing excellent facilities at the laboratories during the time that the material for this report was collected.

SUMMARY

1. The ultrastructure of the opercular epithelium in *Spirorbis moerchi,* with special emphasis on the structure of the pore epithelium in brooding and nonbrooding individuals, has been described.

2. In brooding worms, the epithelium bordering the closed opercular pore consists of tall columnar cells. Each cell is characterized by the presence of long microvilli, apical intercellular spaces, an apical ring of membrane associated microfilaments, and bundles of microfilaments arranged parallel to the longitudinal axis.

3. After larval release, the opercular pore remains open, although not maximally, and the cells bordering the pore, including their microvilli and apical intercellular spaces, are now reduced in height. The cells contain an apical filamentous network and a subapical ring of membrane associated microfilaments, but lack the longitudinal bundles observed in the brooding condition.

4. The role of microfilaments, together with other aspects of the ultrastructure of the opercular epithelium, are discussed.

LITERATURE CITED

Bailey, J. H., 1969. Methods of brood protection as a basis for reclassification of the Spirorbinae (Serpulidae). *J. Linn. Soc. Lond. Zool.*, **48**: 387–407.

Baker, P. C., and T. E. Schroeder, 1967. Cytoplasmic filaments and morphogenetic movement in the amphibian neural tube. *Dev. Biol.*, **15**: 432–450.

Borg, F., 1917. Über die Spirorbisarten Schwedens. *Zool. Bidr. Upps.*, **5**: 15–38.

Bubel, A., 1973. An electron microscope investigation into the cuticle and associated tissues of the operculum of some marine serpulids. *Mar. Biol.*, **23**: 147–164.

Buckley, I. K., 1975. Three dimensional fine structure of cultured cells: possible implications for subcellular motility. *Tissue Cell*, **7**: 51–72.

Burke, J. M., 1974. An ultrastructural analysis of the cuticle, epidermis and esophageal epithelium of *Eisenia foetida* (Oligochaeta). *J. Morphol.*, **142**: 301–320.

Cloney, R. A., 1966. Cytoplasmic filaments and cell movements: epidermal cells during ascidian metamorphosis. *J. Ultrastruct. Res.*, **14**: 300–328.

Coggeshall, R. E., 1966. A fine structural analysis of the epidermis of the earthworm, *Lumbricus terrestris* L. *J. Cell Biol.*, **28**: 95–108.

Conrad, G. W., 1973. Control of polar lobe formation in fertilized eggs of *Ilyanassa obsoleta* Stimpson. *Am. Zool.*, **13**: 961–980.

Elsler, E., 1907. Deckel und Brutpflege bei *Spirorbis*. *Z. Wiss. Zool.*, **87**: 603–642.

Gravier, C., 1923. La ponte et l'incubation chez les annélides polychètes. *Ann. Sci. Nat. Zool. Paris Ser. 10*, **6**: 153–247.

Knight-Jones, P., and E. W. Knight-Jones, 1974. Spirorbinae (Serpulidae: Polychaeta) from South Africa, including three new species. *Mar. Biol.*, **25**: 253–261.

Luft, J. H., 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.*, **9**: 409–414.

Potswald, H. E., 1967. Observations on the genital segments of *Spirorbis* (Polychaeta). *Biol. Bull.*, **132**: 91–107.

Potswald, H. E., 1968. The biology of fertilization and brood protection in *Spirorbis* (*Laeospira*) *moerchi*. *Biol. Bull.*, **135**: 208–222.

Potswald, H. E., 1971. A fine structural analysis of the epidermis and cuticle of the oligochaete *Aeolosoma bengalense* Stephenson. *J. Morphol.*, **135**: 185–212.

Reynolds, E. S., 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.*, **17**: 208–212.

Richardson, K. C., L. Jarett, and E. H. Finke, 1960. Embedding in epoxy resins for ultrathin sectioning in electron microscopy. *Stain Technol.*, **35**: 313–323.

Schroeder, T. E., 1969. The role of "contractile ring" filaments in dividing *Arbacia* eggs. *Biol. Bull.*, **137**: 413–414.

Schroeder, T. E., 1970. The contractile ring. I. Fine structure of dividing mammalian (He La) cells and the effects of cytochalasin B. *Z. Zellforsch.*, **109**: 431–449.

Schroeder, T. E., 1972. The contractile ring. II. Determining its brief existence, volumetric changes, and vital role in cleaving *Arbacia* eggs. *J. Cell Biol.*, **53**: 419–434.

Schroeder, T. E., 1973. Cell constriction: contractile role of microfilaments in division and development. *Am. Zool.*, **13**: 949–960.

Spooner, B. A., M. Yamada, and N. K. Wessells, 1971. Microfilaments and cell locomotion. *J. Cell. Biol.*, **49**: 595–613.

Stanley, H. P., 1967. The fine structure of spermatozoa in the lamprey *Lampetra planeri*. *J. Ultrastruct. Res.*, **19**: 84–99.

Storch, V., and U. Welsch, 1970. Über die Feinstruktur der Polychaeten Epidermis (Annelida). *Z. Morph. Ökol. Tiere*, **66**: 310–322.

Thorp, C. H., 1975. The structure of the operculum in *Pileolaria* (*Pileolaria*) *granulata* (L.) (Polychaeta: Serpulidae) and related species. *J. Exp. Mar. Biol. Ecol.*, **20**: 215–235.

Thorp, C. H., and F. Segrove, 1975. The opercular moult in *Spirorbis spirorbis* (L.) and *S. pusilloides* Bush (Polychaeta: Serpulidae). *J. Exp. Mar. Biol. Ecol.*, **19**: 117–143.

Wessells, N. K., B. S. Spooner, J. F. Ash, M. O. Bradley, M. A. Luduena, E. L. Taylor, J. T. Wrenn, and K. M. Yamada, 1971. Microfilaments in cellular and developmental processes. *Science*, **171**: 135–143.

Reference: *Biol. Bull.,* **152**: 221–232. (April, 1977)

HORMONAL CONTROL OF REPRODUCTION IN *BUSYCON:* LAYING OF EGG CAPSULES CAUSED BY NERVOUS SYSTEM EXTRACTS

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Hormones control many aspects of reproductive behavior and the functioning of reproductive organs in gastropods. In prosobranch gastropods, factors controlling sex reversal, which occurs in some species, and sex organ development have been studied (Le Gall and Streiff, 1975). Neurohormonal factors causing egg-laying behavior have been found in opisthobranch gastropods, such as *Aplysia,* (Arch, 1976, for review) and *Pleurobranchaea* (Davis, Mpitsos, and Pinneo, 1974; Ram, Salpeter, and Davis, 1976), and the pulmonate gastropod *Lymnaea* (Geraerts and Bohlken, 1976), but they have not previously been reported in prosobranchs. To provide a broader taxonomic and functional basis on which to make comparative observations, experiments were undertaken on the dioecious prosobranch *Busycon* to determine whether neurohormones controlling egg-laying behavior were to be found in prosobranchs, and also to investigate their presence in a dioecious animal, since *Aplysia, Pleurobranchaea,* and *Lymnea* are all hermaphrodites.

Busycon is the largest marine gastropod found along the eastern coast of the United States. In Woods Hole, Massachusetts, two species of *Busycon* are found: *B. canaliculatum,* the channeled whelk, and *B. carica,* the knobbed whelk. *Busycon* lays its eggs encased in disciform capsules, which are attached to one another in long strings. The capsules laid by the two species of *Busycon* have different shapes, with *B. carica* laying capsules with smooth sides and *B. canaliculatum* laying capsules with ribbed sides (Magalhaes, 1948).

The behavior involved in forming and laying the egg capsules is very similar to the behavior described by Ankel (1929) for *Nassarius,* another dioecious prosobranch. Briefly, a soft bulb-shaped egg capsule is passed from the female gonopore through a groove in the side of the foot to a gland in the bottom of the foot known as the pedal pore. The capsule is hardened and given its species specific shape in the pedal pore, and at the same time the capsule is glued to the substrate or to a previously laid egg capsule to extend the string.

A preliminary report of some of these data has been published (Ram, 1975).

MATERIALS AND METHODS

All work reported here was done at the Marine Biological Laboratory (MBL), Woods Hole, Massachusetts from July to September, 1975. Specimens of *Busycon canaliculatum* and *Busycon carica* were collected locally by the MBL Supply Department and maintained in individual (27 cm × 16 cm × 10 cm deep) or group aquaria with running sea water at the ambient temperature of 20–25° C. Animals were individually numbered. At the time of dissection, animals were weighed with and without shell; the sexual maturity was estimated by weighing the gonad (Strumwasser, Jacklet, and Alvarez, 1969; Betzer and Pilson, 1974) and calculating the gonad index, *i.e.*, gonad weight/animal weight without shell (Betzer and Pilson, 1974). Animals were generally not sexed until the time of dissection; however, it was usually possible to select females when needed, since the largest animals were almost always females: for *B. canaliculatum* 98% (N = 52) over 300 g (weight with shell) were female, 79% (N = 24) 250–300 g were female, and 55% (N = 36) under 250 g were female.

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To dissect the nervous system, the animal's shell was removed and the central nervous system, except the visceral ganglion, was exposed by cutting through the side of the foot along the line of attachment of the mantle. Several red-pigmented ganglia surround the esophagus (Pierce, 1950). [Abbreviations used in this paper to identify parts of the nervous system are: V, visceral ganglion; CNS, central nervous system not including V; and CNR, CNS minus buccal and pedal ganglia.] A short piece of esophagus with the CNS attached was removed, and connective tissue covering the nervous system was dissected from the CNS. The CNS, including a short piece of esophagus, or individual ganglia, was then homogenized in a motor-driven glass-glass tissue grinder in a bed of ice. When needed, the visceral ganglion was dissected from the thin mantle tissue joining the gills to the rectum.

Tissue was first homogenized as dissected for 30 sec. Next, 1.0 ml phosphate buffer (sodium salt, 0.2 M, pH 7.1) or filtered sea water was added, and the sample was homogenized an additional 30 sec. In most experiments the homogenate was centrifuged at 350–500 × *g* for two min in a hand-driven centrifuge, and the supernatant was decanted. The supernatant was diluted as required with an additional amount of the medium used to homogenize, and 0.5 ml aliquots were drawn into disposable 1.0 ml syringes.

Animals were injected through the side of the foot adjacent to the siphon end of the operculum using a 25 g 0.625 inch needle, which was pushed its full length into the side of the foot. Injections of dye by this route showed it to be effective for injecting fluids into the pedal sinus. In most instances, particular samples were tested on at least two recipients in order to increase the reliability with which the hormone, if present, would be detected.

Modifications of the above procedures, to investigate the active agent's solubility and stability to boiling, are described in the results. To study the sensitivity of the agent to protease, parietal ganglia from several animals were homogenized in filtered sea water to yield five or more parietal ganglia/ml. The homogenate was put on a boiling water bath for ten minutes and then cooled on ice for five minutes. This boiling step was necessary because preliminary experiments showed that activity in a crude, unboiled homogenate was lost in five min at 37° C, presumably as a result of degradation by enzymes in the crude extract. After cooling on ice, the homogenate was centrifuged at 350–500 × *g* for two min, and 0.3 ml aliquots were removed from the supernatant for further treatment. The experimental aliquot was preincubated for five min at 37° C, and then 0.3 ml of protease solution (Protease VI, Sigma from *Streptomyces griseus,* 0.01 mg/ml of sea water, preincubated five min at 37° C) was added. After ten min incubation, the sample was put on a boiling water bath for five min to destroy the protease activity. After cooling, 0.5 ml of filtered sea water was added, and the sample was injected into two recipients. Control aliquots, with sea water instead of protease solution added and either incubated as usual (incubated control) or not incubated at all (cold control), were tested. A control for injection of boiled protease in the experimental extract was done by adding boiled protease solution to an active extract prior to injection (inactive protease control).

RESULTS

Induction of egg-laying behavior by nervous system extracts

Laying of egg capsules could be induced by injection of nervous system extracts of *Busycon* into recipient *Busycon*. Both conspecific and interspecific (between *B. carica* and *B. canaliculatum*) injections were effective. The first injection produced the laying of egg capsules in 15 (40%) out of 38 female recipients (Table I). Fifteen of the 23 animals which failed to lay egg capsules on the first injection were tested on subsequent occasions, and, of these, three animals (20%) laid egg capsules.

Animals which had laid egg capsules at least once nearly always laid egg capsules upon subsequent injections (Table II). Injections of CNS or CNR extracts into these animals caused egg capsule laying on 27 (80%) out of 34 occasions. Only one animal which had laid egg capsules once could not be induced to lay on subsequent occasions. (This animal accounts for two of the failures.) Except where otherwise stated, all of the results described were obtained using animals which had been induced to lay egg capsules at least twice. Control injections of phosphate buffer or sea water into these animals never resulted in egg capsule laying (Table II).

To determine whether the difference between animals which reliably laid egg capsules and those which never laid might be attributed to lack of sexual maturity of the nonlayers, the relationship between egg capsule laying and gonad size was examined. Of the specimens of *B. canaliculatum* tested, six had a gonad weight below 0.9 g and a gonad index less than 0.006, and all six failed to lay egg capsules. Five of these had been tested with extracts which caused at least one other animal to lay capsules. Animals with a gonad weight or gonad index above these figures had a greater than even chance of being reliable egg layers [14 (58%) layers out

TABLE I

Laying of egg capsules after first injection with nervous system homogenate (CNS or CNS + V).

		Animals laying/animals injected		
		Homogenizing buffer		
Donor species	Recipient species	PO_4	Sea water	Total
B. canaliculatum	*B. canaliculatum*	9/24	2/6	11/30
B. canaliculatum	*B. carica*	1/3	0/1	1/4
B. carica	*B. canaliculatum*	0/0	3/4	3/4
Total		10/27	5/11	15/38

TABLE II

Laying of egg capsules by "reliable layers" (animals which had laid egg capsules on at least one previous occasion).

Part of nervous system injected	Animals laying/animals injected Homogenizing buffer PO₄	 Sea water	 Total
CNS + V	6/7	0/0	6/7
CNS	11/14	1/1	12/15
CNR	2/2	7/10	9/12
Total	19/23	8/11	27/34*
Homogenizing buffer alone	0/10	0/6	0/16

* Includes 7/9 injections of *B. canaliculatum* into *B. carica* and 2/2 injections of *B. carica* into *B. canaliculatum*. All others are *B. canaliculatum* into *B. canaliculatum*. These are the results of injections into 12 recipient animals.

of 24 animals for which data are available, Fig. 1]. Five of the ten nonlayers in this group had been tested with extracts which caused other animals to lay egg capsules. Although animals with smaller gonads were thus unlikely to lay egg capsules when injected with active extracts, gonad size was not the sole determinant of capsule laying, since not all animals with large gonads could be induced to lay egg capsules.

The egg capsules laid as a result of extract injection were unusual in three respects. First, capsules laid after extract injection did not contain any eggs,

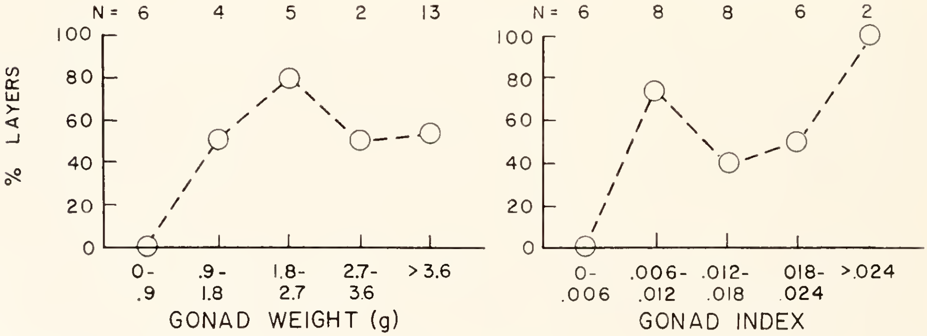

FIGURE 1. Relationship between gonad size and incidence of egg laying of animals injected with nervous system extracts. The graphs illustrate the relationship using two different measurements of gonad size: gonad weight and gonad index (gonad weight/animal weight without shell). Graphs show percentages of animals in various ranges of gonad weight or index which laid egg capsules at least twice. N is the total number of animals in the indicated weight or index range. Data illustrated are from all animals for which complete data were collected, except one animal which laid once and could not be induced to lay again. This animal was at the lower end of the layer distribution (gonad weight = 0.85 g, gonad index = 0.0051).

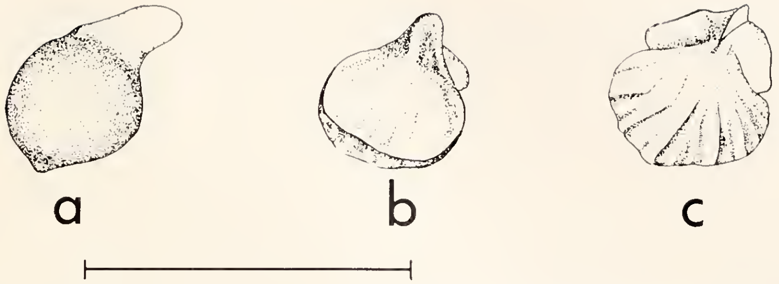

FIGURE 2. Egg capsules laid as a result of nervous system extract injections: (a) soft, bulb-shaped capsule; (b) hardened, well-formed capsule with relatively smooth sides and a double edge laid by *B. carica* injected with *B. canaliculatum* extract; (c) hardened, well-formed capsule with ribbed sides and a single edge laid by *B. canaliculatum* injected with *B. canaliculatum* extract. Similarly shaped capsules were laid by *B. canaliculatum* injected with *B. carica* extract. Scale equals 5 cm.

except in one instance to be discussed later. The active agent will therefore be referred to only as an egg capsule laying substance (ECLS), and not as an egg-laying hormone. Secondly, the capsules often were not well formed and hardened, as spontaneously laid egg capsules usually are. Thirdly, the capsules were, except in one instance, laid individually and were not strung together in strings as are found in the wild.

Figure 2 shows examples of egg capsules laid as a result of extract injections. Most of these egg capsules were soft and bulb-like in appearance (Fig. 2a). Occasionally reasonably well-formed and hardened egg capsules were laid (Fig. 2b, c). The success of the animal in passing the capsule over the edge of the foot into the pedal pore determined whether a soft or hard capsule was produced. On most occasions the capsule slipped out of the groove in the side of the foot, and the resultant capsule was soft and bulb-like. If the capsule was successfully passed to the pedal pore, it was enveloped by the pore for 15 minutes to several hours, after which it emerged as a hardened, well-formed capsule. If a hardened capsule was laid, its shape was typical of the species laying it, regardless of the donor extract (Fig. 2b, c).

The latency between extract injections and the laying of an egg capsule was about two to four hours. If the laying of more than one egg capsule was induced, subsequent capsules appeared at a spacing of about one every three hours.

The usual dosage in these experiments was one-quarter or one-half of the extract of a single CNS. Capsule laying was induced by as little as one-eighth of a CNS extract; however, this was the lowest dose tested, and no systematic investigation of the lowest effective dose was attempted. Injection of greater dosages or several injections of one-quarter CNS dosages, spaced over several hours, frequently resulted in several capsules being laid. The most extreme example of this was a single injection of one CNS + V, which resulted in the laying of six unattached egg capsules over about half a day. Injection of an animal with one-quarter CNS dosages at approximately three hour intervals usually caused an egg capsule to be laid about three hours after each injection. Moreover, injection of extract on the following day would also cause egg capsule laying.

Thus, there appeared to be no refractory period in *Busycon* beyond the time necessary to induce a single egg capsule. This made it possible to use animals repeatedly, though recipients were generally given only one injection per day.

Localization of ECLS within the CNS

ECLS was localized within the nervous system by dissecting parts of the nervous system. In the first set of experiments the usual centrifuged extracts in phosphate buffer were made; in later experiments uncentrifuged sea water extracts were injected. Extracts equivalent to either one-quarter or one-half of the dissected tissue in a whole animal were injected, usually into each of two recipients for each sample. Recipients were chosen by lot, and the identity of injected extracts was concealed until several recipients had laid egg capsules.

In the first series of experiments, with centrifuged phosphate buffer extracts, the parietal ganglia gave the most consistent positive results; seven (100%) out of seven extracts caused the laying of egg capsules. Less consistently, samples containing both the cerebral and pleural ganglia (the demarcation between these ganglia was indistinct, and they were therefore dissected together in this series) also caused the laying of egg capsules [three (60%) out of five extracts]. Extracts containing pedal, buccal or visceral ganglia, or a piece of esophagus did not cause the laying of egg capsules. The results are summarized in Table III.

It seemed possible that laying from cerebral-pleural samples might be caused by contamination by ECLS released during dissection. Therefore, in one of the above experiments, after removing the CNS from the animal, the CNS was frozen under 70% ethylene glycol (Giller and Schwartz, 1971) for further dissection of individual ganglia. The experiment still resulted in both the cerebral-pleural and parietal samples causing the laying of egg-capsules.

It was later discovered (see section on biochemical properties) that a considerable amount of activity was found even in the low speed pellet which was dis-

TABLE III

Localization of egg capsule laying substance within the nervous system (each extract tested on one or two recipients).

	Type of homogenate			
	PO$_4$		Sea water, uncentrifuged	
Part of nervous system tested	A	B	A	B
Parietals	7/7	13/14	6/6	10/10
Cerebrals and pleurals	3/5	5/10	—	—
Cerebrals and left pleural	—	—	4/4	4/4
Right pleural	—	—	4/4	4/5
Pedals	0/5	0/10	2/8	3/16
Buccals	0/5	0/7	—	—
Visceral	0/6	0/10	—	—
Esophagus	0/2	0/4	—	—

A: laying in one or more recipients / extract.
B: animals laying / animals injected.

carded in the above experiments. Therefore, a partial repetition of the localization experiment was done with uncentrifuged seawater extracts. In this set of experiments, the parietal ganglia, the cerebro-pleural ganglia, and one of the pleural ganglia all gave consistent positive results. Pedal ganglia extracts also occasionally gave positive results (Table III). The other ganglia were not tested in this series.

Presence of ECLS in males and females

To test whether ECLS was specific only to the female sex, male and female nervous systems were bioassayed. The CNS was dissected from large males [mean weight with shells = 219 g ± 13 g (s.d.)], and from females of about the same size [mean weight with shells = 210 g ± 18 g (s.d.)]. Extracts were made of individual nervous systems, and the equivalent of one-half of each CNS was injected into each of one or two recipients. On any particular day an equal number of male and female extracts were tested. Recipients were chosen by lot, and the identity of the injected extracts was concealed until several recipients had laid egg capsules.

The results of these experiments were that three (50%) out of six male extracts caused egg capsule laying, and five (83%) out of six female extracts caused egg capsule laying. In other experiments, without the parallel female controls, three out of three male extracts caused egg capsule laying.

Biochemical properties

To gain a better understanding of the ECLS and to provide possible guides for its future purification, experiments were performed on the heat stability, solubility, and protease sensitivity of the agent.

For heat stability, homogenates of CNR in filtered sea water were put on a boiling water bath for five minutes or thirteen minutes or were kept on ice as a control. Samples were subsequently centrifuged at 350–500 × g for two min, and the supernatants were injected into two recipients each. Both boiled extracts caused capsule laying (3/4 recipients), as did controls for each (3/4 recipients). In addition to these positive results, boiled extracts also consistently showed capsule laying activity in the experiments reported below on solubility and protease sensitivity.

For solubility, low speed (350–500 × g for two min) supernatants of CNR homogenized in 0.1 M phosphate buffer were subjected to 105,000 × g for two hours at < 4° C. No ECLS was found in the final supernatant (0/2 extracts, 0/4 recipients), whereas the 105,000 × g pellet contained ECLS (2/2 extracts, 3/3 recipients). Attempts to release ECLS from the 105,000 × g pellet by freeze-thawing or by exposing it to high salt concentrations (1.0 M NaCl for two hours at 0° C) failed; however, boiling the low speed supernatant of a sea water homogenate for ten minutes before centrifugation at 105,000 × g partially solubilized ECLS (one extract: 2/2 recipients of supernatant, 2/2 recipients of pellet).

The partition of the capsule-laying agent in the initial low speed (350–500 × g for two min) centrifugation was also studied. In three experiments using CNR extracts in filtered sea water, capsule laying was produced by three out of three pellets and two out of three supernatants.

Two extracts were tested for the protease sensitivity of ECLS. Samples treated with active protease never caused capsule laying (0/4 recipients), whereas all three control groups caused capsule laying (incubated control, 3/4 recipients; cold control, 4/4 recipients; inactive protease control, 4/4 recipients).

Spontaneous egg-laying

Spontaneous laying of egg capsules was observed on only one occasion. Since the conditions in which this occurred were unique for this laboratory, a description of these conditions may be a possible guide to future investigations of the natural controls of egg laying in *Busycon.*

Animals were usually maintained in individual aquaria; however, on August 29 the reliable layers in the laboratory (15 animals at the time) were put into a large group tank, along with several untested females and three males. When next observed, on September 3, several egg capsules had been spontaneously laid in the tank. Two animals had strings of egg capsules still attached to the foot. These two animals were transferred to individual aquaria. One of them continued to lay egg capsules until a string containing 79 egg capsules in all had been laid. This animal took more than a week to lay its capsule string, laying capsules at a rate of one capsule every three hours, a rate comparable to that seen in the field (Magalhaes, 1948). Capsule laying probably began about midnight on August 30, about 24 hours after the animals had been put in the group tank. The other animal which had an attached capsule string laid only one more capsule (for a total of 14 capsules) after being put into its own aquarium. This animal probably began laying about 48 hours after being put in the group tank. These capsule strings, laid in the laboratory, were identical in appearance to those usually found in the field (illustrated, for example, in Hyman, 1967, p. 304).

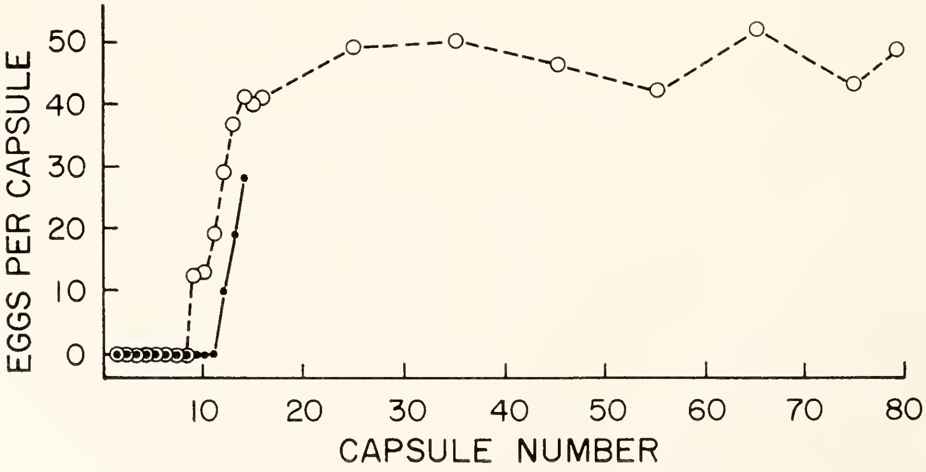

FIGURE 3. Number of eggs in selected capsules of two egg capsule strings laid in the laboratory. Open circles show data from a 79-capsule string; solid circles, data from a 14-capsule string.

The day after removing the two animals with attached capsule strings from the group tank, more spontaneously-laid capsules were found in the tank. At this point, all animals were separated into individual aquaria, and no more capsules were laid spontaneously.

The capsules in the 79-capsule and 14-capsule strings contained eggs. The number of eggs per capsule was counted by cutting open selected capsules in these two strings. These data (Fig. 3) show that the first 10 or so capsules in each string contained no eggs, and then the number of eggs gradually increased until, in the 79-capsule string, the number of eggs per capsule leveled off at about 45. Other strings of spontaneously-laid egg capsules, up to six capsules in length, contained no eggs.

The animal which laid the 79-capsule string was injected with nervous system extracts approximately 3 hours, 8 hours, 11 hours, and 21 hours after the last spontaneously-laid egg capsule was released from the foot. Each of these injections was followed by the laying of a single egg capsule containing eggs. These capsules had 9, 29, not recorded, and 8 eggs in them, respectively.

Discussion

Extracts of the nervous system of *Busycon* contain a substance (ECLS) which causes the laying of egg capsules when injected into female *Busycon,* demonstrating for the first time an agent causing such behavior in a prosobranch gastropod. ECLS may be a hormone which normally causes the laying of egg capsules; however, proof of this would require the demonstration of physiological concentrations of ECLS in the blood during normal laying of egg capsules. Like the egg-laying hormones of the opisthobranchs *Aplysia* (Toevs and Brackenbury, 1969) and *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976), ECLS is sensitive to proteolytic enzymes, and therefore likely to be a polypeptide. It is also similar to the egg-laying hormones of *Aplysia* (Kupfermann, 1970) and *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976) in being stable to boiling.

In contrast to the egg-laying hormones of *Aplysia* and *Pleurobranchaea,* which cause the laying of egg ribbons with eggs, ECLS usually causes the laying of egg capsules which do not contain eggs. It may be that ECLS causes the insertion of eggs into egg capsules, but only when it has been working over longer periods than were used in these experiments. The lack of eggs in the first 8–11 capsules laid by spontaneous layers (Fig. 3) may similarly be explained if ECLS must be secreted for up to a day before egg-containing capsules can be laid. Consistent with this explanation is the observation that ECLS caused laying of egg-containing capsules when injected into an animal that had already been laying eggs for several days.

Other explanations for the lack of eggs in capsules laid after ECLS injection are possible. For example, another agent, not found in the central nervous system, may be necessary. Perhaps eggs cannot be inserted unless they are first fertilized, as may have happened in the spontaneous layers which had been placed originally in a group tank. It is fairly evident, however, that lack of maturity of recipients is not an explanation for the lack of eggs. The gonad indices of the two animals which spontaneously laid capsules with eggs were 0.0096 and 0.0181, which is in the middle of the range of animals laying capsules (0.006 to 0.025, Fig. 1).

As in similar studies, on *Aplysia* (Strumwasser *et al.,* 1969), *Pleurobranchaea* (Ram, Salpeter, and Davis, 1977) and *Lymnaea* (Geraerts and Bohlken, 1976), not all animals could be induced to lay eggs or egg capsules. Fluctuations in the amount of hormone in the injected extract in *Aplysia* and *Pleurobranchaea* could not be eliminated as an explanation in those studies, because active extracts were never tested systematically on several recipients. In the present study, all but six of the nonlayers were tested with extracts that caused laying in at least one other recipient. Thus, factors within the recipient are responsible for this lack of response. Geraerts and Bohlken (1976) reached a similar conclusion in *Lymnaea.* In *Busycon,* one of these factors is gonad size. All six animals with gonad indices below 0.006 failed to lay capsules when injected with extract known to be active. Other factors, presently unknown, prevent some animals with gonad indices above this size from laying capsules.

Experiments on the localization of ECLS-containing cells indicate that the hormone was most consistently found in the parietal ganglia, but was also found in the cerebral and pleural ganglia. ECLS thus appears to be more widely distributed throughout the nervous system than the egg-laying hormone of *Pleurobranchaea* (Ram, Salpeter, and Davis, 1976), *Aplysia* (Strumwasser *et al.,* 1969), and *Lymnaea* (Geraerts and Bohlken, 1976, and Geraerts, personal communication). ECLS-synthesizing cell bodies may not be as widely distributed as this, since the hormone in some ganglia may be contained in processes which have their cell bodies elsewhere. The parietal ganglia, which gave the most consistently positive results, would seem to be the most likely site for the cell bodies producing ECLS. Hoffmann (1936) indicates that the bag cells of *Aplysia,* which synthesize its egg-laying hormone (Arch, 1976), are homologous to parietal ganglia; and Ram, Salpeter, and Davis (1977) propose that cells in the pedal ganglia of *Pleurobranchaea* which contain its egg-laying hormone are also homologous to parietal ganglia. Thus, the hormones in these animals and the cells synthesizing them may be homologous.

Similarities in some biochemical properties (stability to boiling and sensitivity to protease) have already been noted. ECLS appears to be less soluble than the egg-laying hormones of either *Aplysia* or *Pleurobranchaea;* however, even the latter appears to lose some activity into a low speed pellet (Ram, unpublished data). The insolubility of ECLS prior to boiling may be artifactual, but a more interesting possibility is that the hormone may be bound in vesicles or to a neurophysin which is denatured by boiling. Whatever the function of this insolubility for *Busycon,* it appears useful in purifying the hormone. Soluble proteins can be removed prior to boiling, and boiling can then be used to solubilize ECLS and probably a few other proteins.

ECLS was found in the nervous system of both male and female *Busycon.* While these experiments do not prove that the male ECLS is identical to the female ECLS, the presence in males of a hormone which in females controls a female function, such as egg-laying, is not unusual. In the octopus, the optic glands contain a hormone, which is identical in both sexes, and which stimulates vitellogenesis in females and spermatogenesis in males (Richard, 1970). In both the starfish (Kanatani and Ohguri, 1966) and the polychaete *Arenicola* (Howie, 1966), maturation of eggs and egg-laying are caused by agents found in both sexes. Prolactin is known to have many functions besides promoting mammary growth and milk secretion in female mammals, for which it was named, and it occurs in both sexes of mammals as well as in amphibians, birds, reptiles, and teleost fish (Bern and Nicoll, 1968). Thus, it is a general principle that these "female" hormones occur in both sexes, and the discovery of ECLS in both sexes of *Busycon* supports this principle. The role of ECLS in male *Busycon* is unknown.

Further study is needed on the natural controls of egg-laying in *Busycon* and other gastropods. While it is known that a certain degree of sexual maturity must be attained before egg-laying behavior can be induced by hormone injection, it is not known why some seemingly mature animals cannot be induced to show any egg-laying behavior (Fig. 1, and similar analyses by Strumwasser *et al.,* 1969; Ram, Salpeter, and Davis, 1977). Moreover, environmental stimuli which are immediate causes of egg-laying in these animals are unknown. The almost simultaneous initiation of "spontaneous" egg-laying in *Busycon* when put in a group tank and its cessation upon separating the animals suggest that an analysis in *Busycon* may be possible.

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SUMMARY

Mature specimens of female *Busycon* laid egg capsules when injected with extracts of nervous systems of male or female *Busycon.* The substance causing this behavior, named egg capsule laying substance (ECLS), was found most reliably in parietal ganglia, less consistently in cerebral-pleural ganglia, and rarely in other ganglia. Both species of *Busycon* found in Woods Hole, *B. canaliculatum* and *B. carica*, contained ECLS, and ECLS of each species was active in the other.

ECLS activity was not destroyed by boiling for up to fifteen minutes. Centrifugation of nervous system extracts at 105,000 × g yielded ECLS only in the pellet. ECLS was not released from the pellet by freeze-thawing or by 1.0 M NaCl, but could be partially solubilized by boiling extracts before centrifugation. ECLS activity was destroyed by protease.

Several animals "spontaneously" laid strings of egg capsules after being put in a group tank with males and other females. Approximately the first ten capsules laid by these animals were devoid of eggs, after which egg-containing capsules were laid. Injection of ECLS into a spontaneous egg layer within a few hours after cessation of spontaneous egg laying caused the laying of capsules containing eggs. The possibility that ECLS may normally be responsible for the laying of both eggs and egg capsules is discussed.

LITERATURE CITED

ANKEL, W. E., 1929. Bildung der Eikapsel bei Nassa-Arten. *Zool. Anz. Suppl.,* **4**: 219–229.

ARCH, S., 1976. Neuroendocrine regulation of egg laying in *Aplysia californica. Am. Zool.,* **16**: 167–175.

Bern, H. A., and C. S. Nicoll, 1968. The comparative endocrinology of prolactin. *Recent Prog. Horm. Res.*, **24**: 681–720.

Betzer, S. B., and M. E. Q. Pilson, 1974. The seasonal cycle of copper concentration in *Busycon canaliculatum* L. *Biol. Bull.*, **146**: 165–175.

Davis, W. J., G. J. Mpitsos, and J. M. Pinneo, 1974. The behavioral hierarchy of the mollusk *Pleurobranchaea*. II. Hormonal suppression of feeding associated with egg laying. *J. Comp. Physiol.*, **90**: 225–243.

Geraerts, W. P. M., and S. Bohlken, 1976. The control of ovulation in the hermaphroditic freshwater snail *Lymnaea stagnalis* by the neurohormone of the caudodorsal cells. *Gen. Comp. Endocrinal.*, **28**: 350–357.

Giller, E., Jr., and J. H. Schwartz, 1971. Choline acetyltransferase in identified neurons of abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.*, **34**: 93–107.

Hoffman, H., 1936. Das Nervensystem. Pages 640–840 in H. G. Bronns, Ed., *Klassen und Ordnungen des Tierreichs. Opisthobranchia.* (3. Band: Mollusca, II. Abteilung: Gastropoda, 3. Buch: Opisthobranchia, Teil 1). Akademische Verlagesgesellschaft M. B. H., Leipzig.

Howie, D. I. D., 1966. Further data relating to the maturation hormone and its site of secretion in *Arenicola marina* Linneaus. *Gen. Comp. Endocrinol.*, **6**: 347–361.

Hyman, L., 1967. *The Invertebrates: Mollusca I (Vol. VI)*. McGraw-Hill Book Company, New York, 792 pp.

Kanatani, H., and M. Ohguri, 1966. Mechanism of starfish spawning. I. Distribution of active substance responsible for maturation of oocytes and shedding of gametes. *Biol. Bull.*, **131**: 104–114.

Kupfermann, I., 1970. Stimulation of egg laying by extract of neuroendocrine cells (bag cells) of abdominal ganglion of *Aplysia*. *J. Neurophysiol.*, **33**: 877–881.

Le Gall, S., and W. Streiff, 1975. Protandric hermaphroditism in prosobranch gastropods. Pages 170–178 in R. Reinboth, Ed., *Intersexuality in the animal kingdom*. Springer-Verlag, New York.

Magalhaes, H., 1948. An ecological study of snails of the genus *Busycon* at Beaufort, North Carolina. *Ecol. Monogr.*, **18**: 377–409.

Pierce, M. E., 1950. *Busycon canaliculatum*. Pages 336–344 in F. A. Brown, Jr., Ed., *Selected invertebrate types*. John Wiley and Sons, Inc., New York.

Ram, J. L., 1975. Laying of egg capsules in *Busycoptypus* induced by nervous system extracts. *Biol. Bull.*, **149**: 443.

Ram, J. L., S. Salpeter, and W. J. Davis, 1976. Behavioral effects, biochemistry, and species specificity of a molluscan hormone. Pages 90–91 in *Abstracts, Fifth International Congress of Endocrinology*. Bruhlsche Universitatsdruckerei, Giessen, Germany.

Ram, J. L., S. R. Salpeter, and W. J. Davis, 1977. *Pleurobranchaea* egg-laying hormone: localization and partial purification. *J. Comp. Physiol.*, **114**: in press.

Richard, A., 1970. Differenciation sexuelle des cephalopodes en culture *in vitro*. *Annee Biol.*, **9**: 409–415.

Strumwasser, F., J. W. Jacklet, and R. B. Alvarez, 1969. A seasonal rhythm in the neural extract induction of behavioral egg laying in *Aplysia*. *Comp. Biochem. Physiol.*, **29**: 197–206.

Toevs, L. A., and R. W. Brackenbury, 1969. Bag cell-specific proteins and humoral control of egg laying in *Aplysia californica*. *Comp. Biochem. Physiol.*, **29**: 207–216.

Reference: *Biol. Bull.*, **152**: 233–250. (April, 1977)

THE ONTOGENY OF SWIMMING BEHAVIOR IN THE SCYPHOZOAN, *AURELIA AURITA*. I. ELECTROPHYSIOLOGICAL ANALYSIS[1]

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The life cycle of a scyphozoan coelenterate typically includes several developmental stages with strikingly different morphologies and distinct behavioral repertoires. The large, motile medusae produce gametes which, upon fertilization, develop into planulae larvae. Planulae settle on a substrate and grow into sessile polyps termed scyphistomae. Scyphistomae feed and produce other scyphistomae by budding. Seasonally the scyphistomae produce new medusae by transverse fission. A series of grooves forms perpendicular to the oral-aboral axis and the segments formed metamorphose into larval medusae called ephyrae. Development of ephyrae proceeds in an oral-aboral direction on the polyp, the most oral segment is the most advanced and is the first to be released as a free ephyra. This process of medusa production is termed strobilation, and the transversely segmented polyp is called a strobila. The aboral portion of the strobila, where segmentation has not occurred, develops tentacles. When all the ephyral segments have been released the remaining polyp feeds and repeats the strobilation process the following season. The released ephyrae develop into large, sexual medusae, completing the life cycle.

Behavior of scyphozoans has been most extensively studied in the adult medusae, with particular attention having been given to the control of the rhythmic swimming pulsations. These pulsations are initiated by marginal ganglia (rhopalia) located in the periphery of the bell (Romanes, 1876). Spontaneous discharges from pacemakers of the rhopalia spread through a rapidly-conducting nerve net (giant fiber nerve net, GFNN) located in the subumbrellar ectoderm. GFNN activity evokes contractions of the swimming muscles and coordinates the swimming beat (Romanes, 1876; Schäfer, 1879; Horridge, 1953, 1954, 1956a). The swimming muscles of medusae are striated with the characteristic birefringent and nonbirefringent pattern seen in the muscles of more advanced animals. There is also a diffuse, more slowly-conducting nerve net (diffuse nerve net, DNN), which modulates pacemaker activity through polarized synapses and also serves to control marginal tentacle activity (Romanes, 1877; Horridge, 1956a, 1959; Passano, 1965).

The behavior of a newly-released ephyra differs somewhat from that of adult medusae. The released ephyra exhibits three behavioral responses of importance: first, rhythmic swimming involves the simultaneous folding of the eight arms toward the mouth; secondly, feeding involves the coordinated movement of the manubrium and an individual arm; and thirdly, spasm, which occurs during strong stimulation, involves all the arms as in swimming, but the contraction is tonic and can last several minutes (Horridge, 1956a). Physiological analysis of these three responses in company with histological examination has demonstrated the presence of two conducting systems controlling ephyral behavior. The GFNN propagates the swimming beat while the DNN is responsible for feeding and spasm (Horridge, 1956a). The ephyral responses, feeding and spasm, are not observed in adult medusae, although both medusae and ephyrae possess diffuse and giant fiber nerve nets.

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Chapman (1965, 1966) has described the behavior of scyphozoan polyps and the organization of the musculature associated with that behavior. Scyphistomae of *Aurelia aurita* have no striated muscles, show no spontaneous activity, and respond only sluggishly to mechanical stimulation. Prey is captured by nematocysts on the tentacles and transferred to the mouth by tentacular bending. Contact between the mouth and tentacles during feeding is followed by mouth opening. Chapman proposes that the responses of the polyp are coordinated mechanically rather than neuronally. In this view a localized response in one part of the animal mechanically stimulates and causes a response in another part of the animal. There is a nerve net in *A. aurita* polyps, but neither sensory nor pacemaker cells have been morphologically identified (Chapman, 1965). Although electrophysiological techniques have been used successfully in other coelenterate polyps (*e.g.*, Josephson, 1965; Josephson and Macklin, 1969; McFarlane, 1969; Morin and Cooke, 1971; Rushforth, 1971; Ball, 1973; Ball and Case, 1973; Spencer, 1974a; Stokes, 1974; early work summarized in Bullock & Horridge, 1965), these have not previously been used with scyphozoan polyps.

During strobilation, polyp behavior becomes transformed into that of the medusa. The ephyrae attached to a strobila begin beating well before their release, and each beats independently of its neighbors. Autonomy of behavior in a strobila is not complete however, since the entire strobila will shorten when stimulated (Horridge, 1956a).

The transformation of a sessile, phlegmatic polyp to an active, swimming medusa is dramatic. During the transformation the polyp develops clear neurological control mechanisms; most obvious are the spontaneously active pacemakers interconnected by a fast, through-conducting nerve net. Thus at the oral end of the strobila are beating ephrae with pacemakers and giant fiber nerve nets coordinating the striated swimming muscles, while at the aboral end of the strobila is a tentaculate polyp lacking spontaneous activity, obvious neurological coordination, or striated muscles. The strobila, then, is a chimera of the morphology and behavior of both polyp and medusa presented in a developmental gradient along the oral-aboral axis. The purpose of this investigation is to take advantage of the developmental gradient expressed by the strobila and investigate the ontogeny of swimming behavior in a scyphozoan medusa. Particular attention is given to the tentacular behavior of the polyp, since the tentacles of the polyp are developmentally homologous with the rhopalia of medusae (Thiel, 1966), and it is the rhopalia which initiate the swimming beats in medusae. This paper describes the electrophysiological changes which occur during the metamorphosis of the polyp into a strobila. A second paper will consider the physiological changes which occur during the transformation of the polyp to medusa by describing the differential responses of the different stages of the life cycle to ions and drugs.

MATERIALS AND METHODS

Medusa

Medusae of *Aurelia aurita* (5–8 cm in diameter) were collected from the Eel Pond, Woods Hole, Massachusetts. Collected medusae were kept in a deep tank with slowly flowing sea water until they were used.

Suction electrodes were used to record the spontaneous pacemaker potentials in the marginal ganglia. Fine electrode tips were made by pulling 1 mm glass capillary tubing on a standard micro-electrode puller, breaking the tips, grinding them flat on a dry Arkansas oil stone, and fire polishing the tips with a micro-burner to a 50 μm opening. This procedure produced smooth, flat electrode tips which minimized tissue damage and provided a tight seal.

Marginal ganglion activity and the associated mechanical swimming contractions were recorded simultaneously from a 45° segment of a medusa. The tissue wedge was excised by cutting along the adradii adjacent to a marginal ganglion and pinned aboral-side up in a constant-temperature chamber maintained at the same temperature as the animal holding tank (15–18° C). A hook placed through the mesoglea at the apex of the wedge was attached to a force-displacement transducer to record contractions. Potentials from marginal ganglia were recorded with a glass suction electrode attached to the aboral surface of the rhopalium near the ocellus. Potentials were recorded between the suction electrode and an indifferent, Ag/AgCl bath electrode. Recorded activity was amplified by a high gain AC-amplifier with a long-time constant. Both mechanical and electrical events were displayed on a penwriter. Marginal ganglion potentials from isolated ganglia were recorded in a similar manner.

Scyphistoma

Scyphistomae of *A. aurita* were obtained from the Supply Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, and also raised from planulae released from medusae of *A. aurita* collected from the Eel Pond. Some additional polyps (Woods Hole strain) were obtained from Dr. Dorothy Spangenberg (University of Colorado, Boulder, Colorado).

Strobilation of scyphistomae usually occurs in the presence of I^-. To prevent strobilation, the scyphistomae were maintained in I^--free artificial sea water (ASW). Individual polyps were isolated in, and allowed to attach to, 13 × 9 mm test containers which were placed in storage dishes containing 250 ml I^--free ASW prepared by passing ASW (463.0 mM Na^+, 9.9 mM Ca^{+2}, 9.7 mM K^+, 51.2 mM Mg^{+2}, 538.6 mM Cl^-, 27.0 mM SO_4^{-2}, and 2.3 mM HCO_3^-) through an ion exchange resin (AG1-X2, BioRad Laboratories; see Spangenberg, 1971). The test containers with the isloated polyps could be removed from the storage dishes either for exchanging the culture medium or experimental purposes without disturbing the polyps. All polyps were kept at 4° C and fed twice weekly with newly hatched *Artemia salina* nauplii. Several hours after feeding the culture, the medium was replaced with fresh I^--free ASW to remove uningested nauplii.

Polyps were electrically stimulated by platinum pin electrodes, 75 μm in diameter, insulated to the tip with teflon, and mechanically stimulated with a hair attached to a glass rod. Suction electrodes, similar to that used with medusae, were used to record electrical activity.

Tentacle activity was detected by a fiber optics probe which monitored movement without contact with the animal. The probe was constructed by passing a 50 cm long × 200 μm fiber optics light guide down the bore of an L-shaped, 1 mm O.D. glass capillary tube so that one end of the light guide protruded 1 mm from the short end of the "L". Two similarly prepared light guides were attached to a glass rod so that the short ends of the "L"s were pointing at each other. Light from a microscope illuminator was passed down one light guide, and the free end of the second guide was inserted into a light tight box containing a selenium photovoltaic cell. The tips of the light guides were 1 mm apart and aligned for maximum output, then placed over a polyp so that a tentacle came between the tips of the probe. Since the tentacle is translucent, any slight movement altered the amount of light reaching the photocell. The shape and amplitude of the photocell output were not necessarily linearly related to tentacle movement, but the output of the photocell gave an accurate representation of the onset of movement. The output of the photocell was amplified and displayed on one channel of an oscilloscope or penwriter, while the potentials recorded from the same tentacle as the mechanical events were displayed on another recording channel.

The fiber optics proble could not be situated to detect lip movements. Lip behavior in the immediate area of the suction electrode was monitored visually and was recorded along with the electrical events by manually triggering an event marker on the penwriter.

Strobila

Polyps of *A. aurita* attached to individual test containers and cultured in I^--free ASW, as described previously, were induced to strobilate by replacing the I^--free ASW with 10^{-5} M KI in ASW and continuing all other procedures (Spangenberg, personal communication). Strobilation occurred in 6–8 weeks in all cultures so treated.

The mechanical and electrical recording methods used on the scyphistoma were also used on the strobila. Experiments on both scyphistoma and strobila stages were carried out in a constant temperature chamber maintained at 4° C.

RESULTS

Medusa

As has been previously reported (Horridge, 1953, 1954), in medusae there is a one-to-one correspondence between the potentials recorded from a marginal ganglion and swimming contractions (Fig. 1A). A contraction of the swimming muscles follows a marginal ganglion potential (MGP) after a latency of approximately 300 msec. MGPs from isolated ganglia are produced at regular intervals for extended periods of time (Fig. 1B).

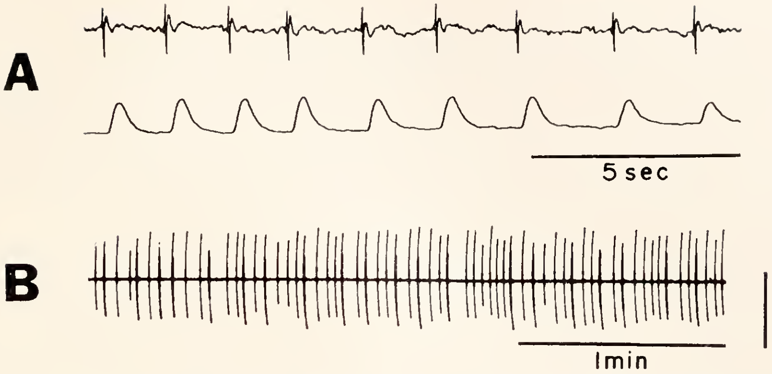

FIGURE 1. Electrical activity and behavior of adult medusae. A, portion of a record from a 45° segment of a medusa; the upper trace shows marginal ganglion potentials (MGPs), the lower trace is a simultaneous recording of mechanical activity. B, MGPs from an isolated marginial ganglion. Voltage calibration is 20 mV.

Scyphistoma

Several types of spontaneous polyp movements were observed in the absence of any apparent stimulation. During an extended observation period most of the tentacles can be seen to contract at one time or another in no set pattern. Each tentacle, when it contracts, shortens without bending and, after a time, slowly elongates. Sometimes the tentacle shortening is the result of a series of small contractions, the whole process occurring in one or two seconds. A single contraction of a restricted, localized portion of a tentacle may occur, resulting in either a shortening, a bending, or at times, the forming of a loop. Bending of an individual tentacle on the oral side brought the tentacle toward the mouth or completely into the mouth and is typical of the feeding response. If the tentacle was inserted into the mouth, a short time later the tentacle would be retracted from the coelenteron with bending occurring at the base of the tentacle followed by the relaxation of the muscles on the oral side. This tentacle behavior was the only one which was associated with mouth behavior. Infrequently a tentacle, in the normal extended position, bent aborally by contracting aboral muscles at the base of the tentacle. Rarely, the tentacles were bent completely parallel to the column. Occasionally all of the tentacles contracted simultaneously. In this behavior the tentacles also bent simultaneously at the bases causing the tentacle tips to meet at the oral-aboral axis above the mouth forming an arch over the oral disk.

Two types of spontaneous lip behavior were seen, localized retraction and flaring. During a flare the entire lip retracts causing the mouth to gape. Both lip behaviors can occur in the absence of tentacular activity; however, lip flaring also occurs as a tentacle bends toward the mouth during feeding as mentioned above.

Spontaneous activity of the column is infrequent. The column can shorten either symmetrically or asymmetrically. Complete symmetrical shortening of the column results from a series of short symmetrical contractions. Symmetrical contractions of the column were always accompanied by the simultaneous shortening and folding of the tentacles over the mouth. This behavior is probably a protective response.

The synchrony of tentacles and column contractions in this response indicates that the protective response is coordinated.

Electrical stimulation of the tentacles caused only the contraction of the individual tentacle stimulated. Similarly, the only effect resulting from electrical stimulation of the lip was a localized retraction of the lip in the area stimulated. Symmetrical column shortening could be initiated by stimulating any portion of the animal but required stimuli considerably stronger than that used to initiate tentacle contractions.

Gentle mechanical stimulation with a hair usually did not elicit a response from either the lip or tentacles. In those cases where a response occurred, the tip would retract locally, or the stimulated tentacle would shorten and occasionally bend toward the mouth and insert into the coelenteron. Once, when a tentacle was touched on the external side, it responded with a localized contraction and bent aborally. Vigorous mechanical prodding of the column never caused a response; however, such stimuli on the tentacles often initiated the protective response.

Electrical potentials were detected in the tentacles and lip, but not in the column (Fig. 2A). Potentials recorded from the tentacles were associated with tentacle contractions and therefore termed tentacle contraction potentials (TCPs). The term is only used here to identify the tissue from which the potentials were recorded and does not imply that the potentials recorded from scyphistomae are homologous with tentacle potentials observed in the hydrozoa (Passano and McCullough, 1962; Rushforth and Burke, 1971; Morin and Cooke, 1971; Ball, 1973).

Figure 2B shows a simultaneous recording of TCPs and tentacular movement. Characteristically, potentials occur 60 to 120 msec prior to the onset of tentacular contractions and are conducted along the length of the tentacle (Fig. 2C). A TCP has a conduction velocity of about 4 mm/sec, determined by the difference in arrival times between two suction electrodes placed along the length of a tentacle. Potentials in a tentacle were always independent of the electrical activity in other tentacles; therefore, there appears to be no conduction of TCPs between tentacles.

Potentials recorded from the lip were always associated with a localized retraction of the lip. Simultaneous recordings from two points in the lip indicate that potentials occur independently and correspond to movements only under the recording electrode; no conduction was found between the two recording points (Fig. 2D). Flaring did not occur when the suction electrodes were attached to the lip, so electrical potentials associated with flaring have not yet been recorded.

Strobila

The onset of strobilation is indicated by an elongation of the polyp and the appearance of a groove marking the first ephyral primordium (Fig. 3). The polypoid tentacles still remain but are being absorbed. Marginal ganglia develop at the bases of the perradial and interradial tentacles and first appear as bulges at the bases of the tentacles. Adradial tentacles do not show these bulges and some have been eliminated in Figure 3 for clarity.

Electrical potentials recorded from the tentacles at this stage were always associated with tentacle contractions and appear to be the same as TCPs occurring in scyphistomae (Figure 3A). As with the scyphistoma, there was no conduction

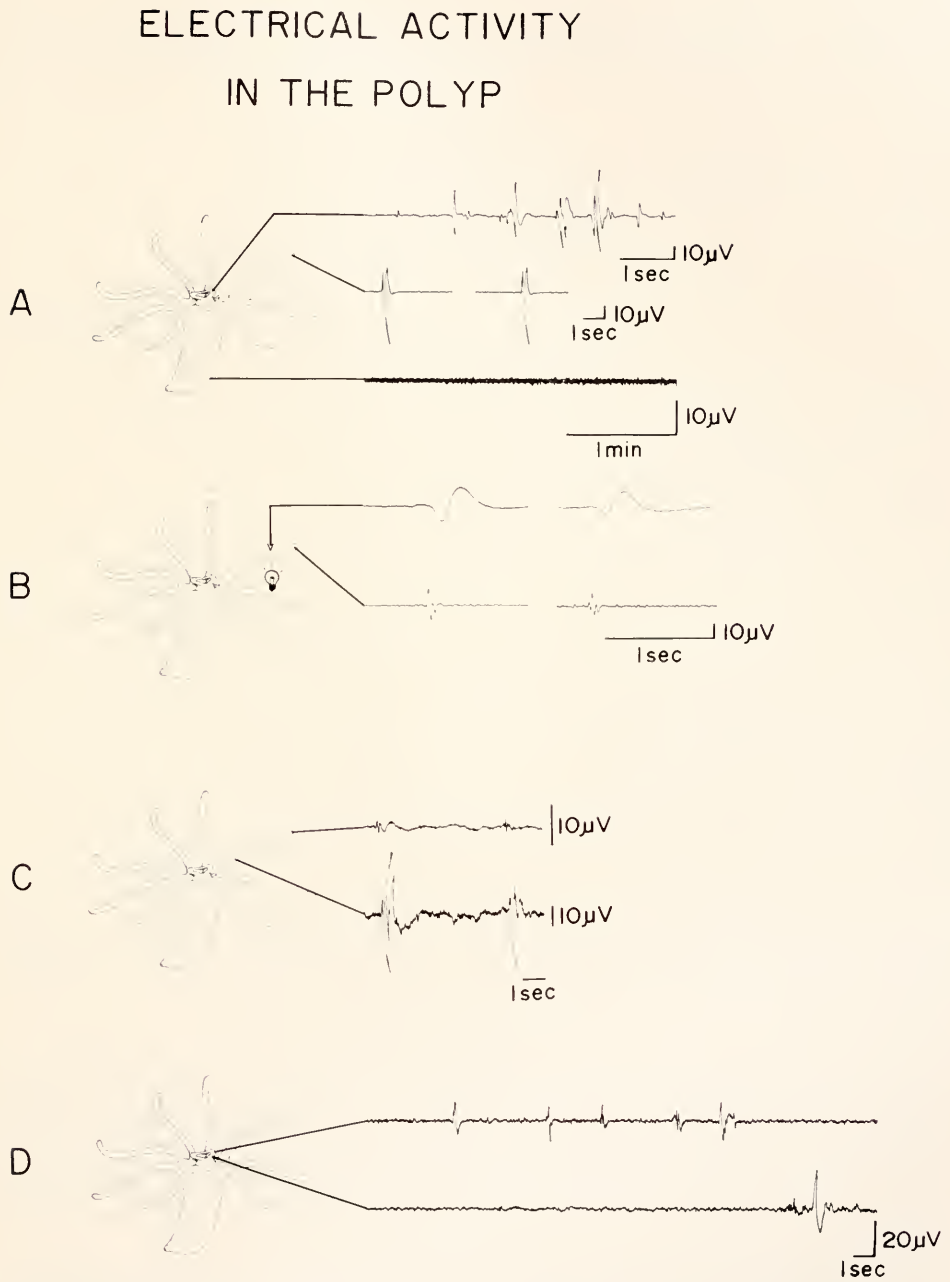

FIGURE 2. Spontaneous electrical activity in the scyphistoma. A, electrical recordings from the tentacles, lip, and column; B, electrical activity in a tentacle (lower record) and the associated movement recorded with the photoelectric-movement detector (upper record); C, conduction of the tentacle contraction potential (TCP); D, non-conduction of lip potentials.

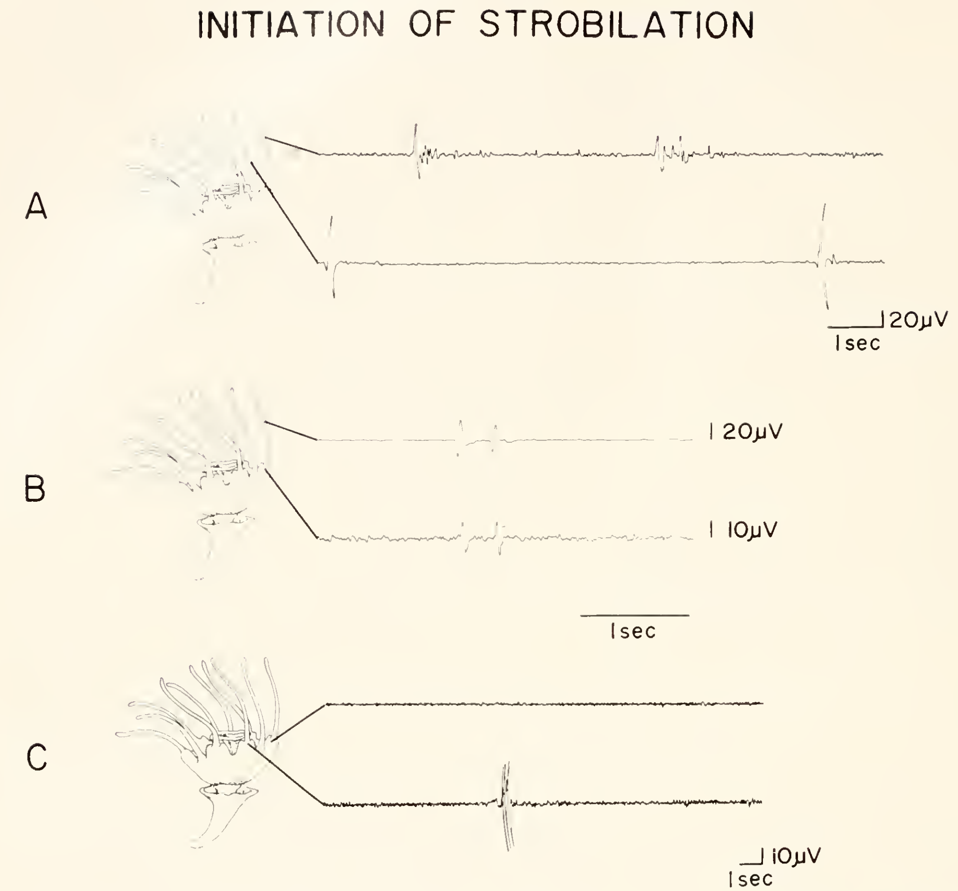

FIGURE 3. Electrical activity in an early strobila (no beating observed). A, TCPs recorded in two tentacles, one rhopalial and the other non-rhopalial; B, portions of records showing TCP conduction along a tentacle; C, electrical recordings between two rhopalial *enlagen* showing the absence of conduction in this early stage of strobilation.

between the tentacles (Fig. 3A). Each TCP was initiated distally and was conducted the length of the tentacle (Fig. 3B). Although marginal ganglion potentials are conducted to other marginal ganglia in the medusa (Passano, 1965), recordings from pairs of marginal ganglia primordia in the strobila demonstrate that TCPs were not conducted between them (Fig. 3C). After the tentacles have receded, TCPs also disappear and for a time the marginal ganglion primordia are electrically quiet.

As strobilation proceeds, the terminal ephyra begins to beat. Simultaneous recording of electrical potentials from a marginal ganglion and mechanical activity in the same arm demonstrated that the potentials corresponded one-to-one with beating movements of that arm and, therefore, are MGPs (Fig. 4A). However, at this early stage of strobilation the MGPs are not always conducted to the other marginal ganglia (Fig. 4B). As development proceeds, a stage is reached where all MGPs are conducted to other ganglia as in the medusa (Fig. 4C). Once beating is established no particular pacemaker appears as the driving pacemaker. By

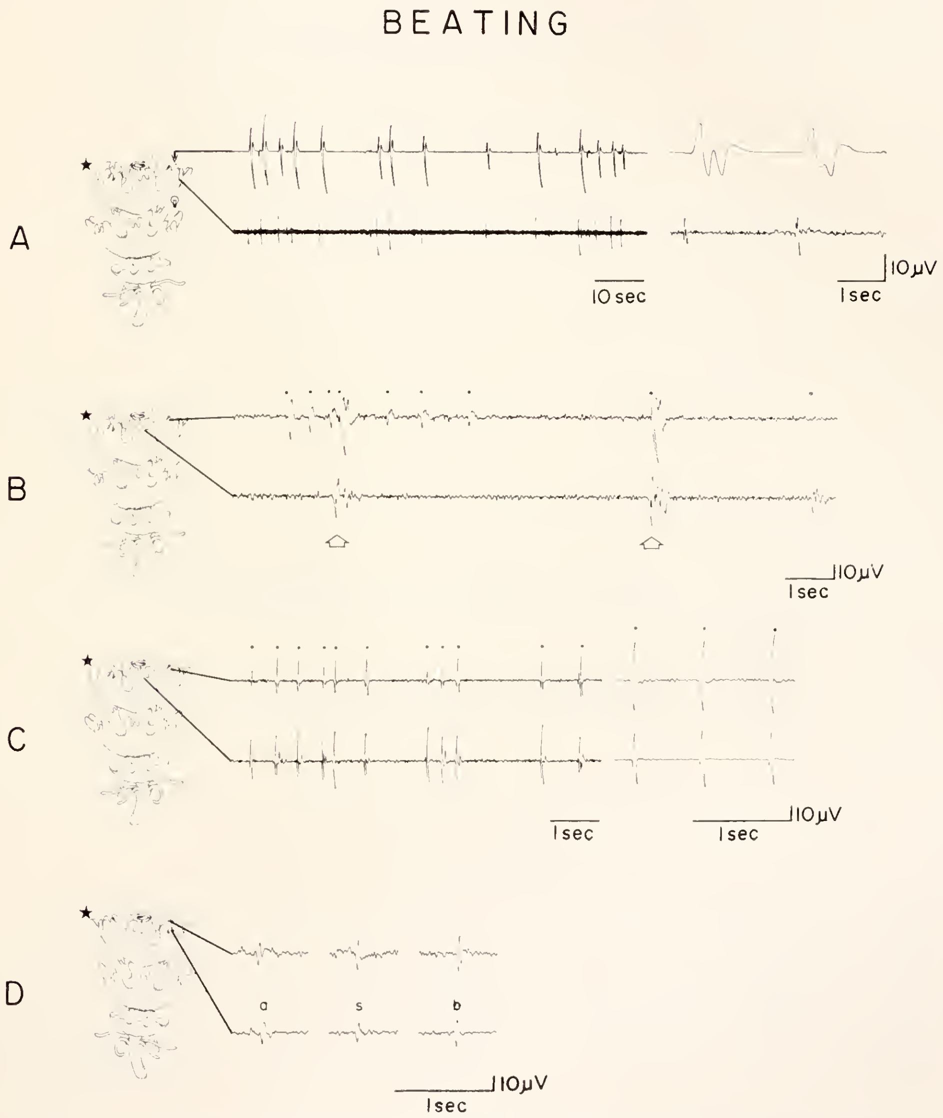

FIGURE 4. Electrical and mechanical activity in attached beating ephyrae. A, upper record, mechanical record of beating activity and in the lower record, the associated marginal ganglion potentials (MGPs); B, conducted (arrows) and nonconducted MGPs; C, conduction of MGPs fully established; D, shifting pacemaker dominance (see text for details). Dots indicate potentials associated with an observed beating contraction. The star indicates a beating ephyral bud.

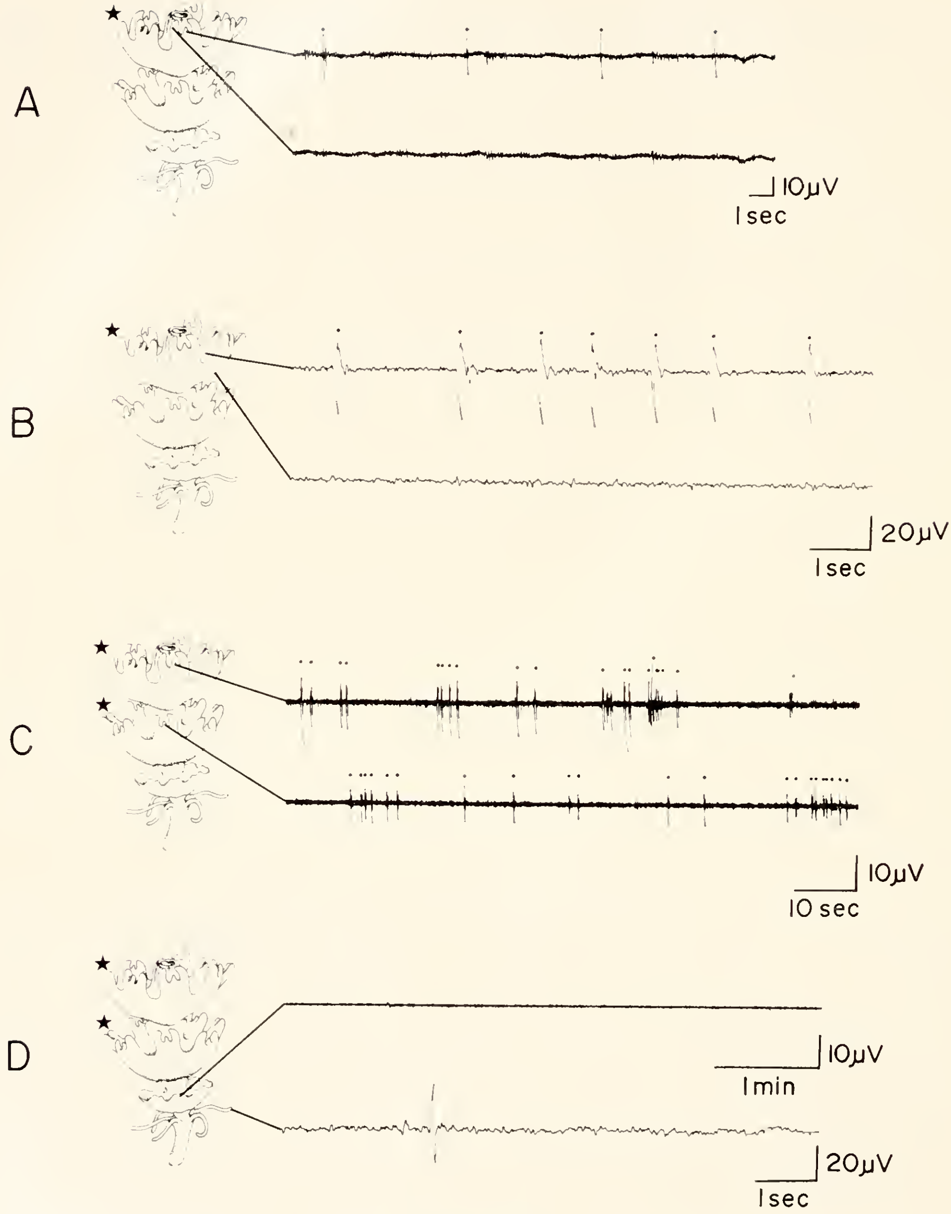

FIGURE 5. Electrical activity from different parts of the strobila. A, electrical records from a marginal ganglion (upper record) and from a lappet (lower record); B, upper record, electrical record from the subumbrella, and the lower record, from the exumbrella; C, marginal ganglion activity from two different attached and beating ephyrae indicating that MGPs are not conducted between ephrae; D, electrical records from a nonbeating ephyra (upper record) and a tentacle from a resting polyp, the tentacle shows a TCP. Dots indicate potentials associated with observed beating contraction. The star indicates a beating ephyral bud.

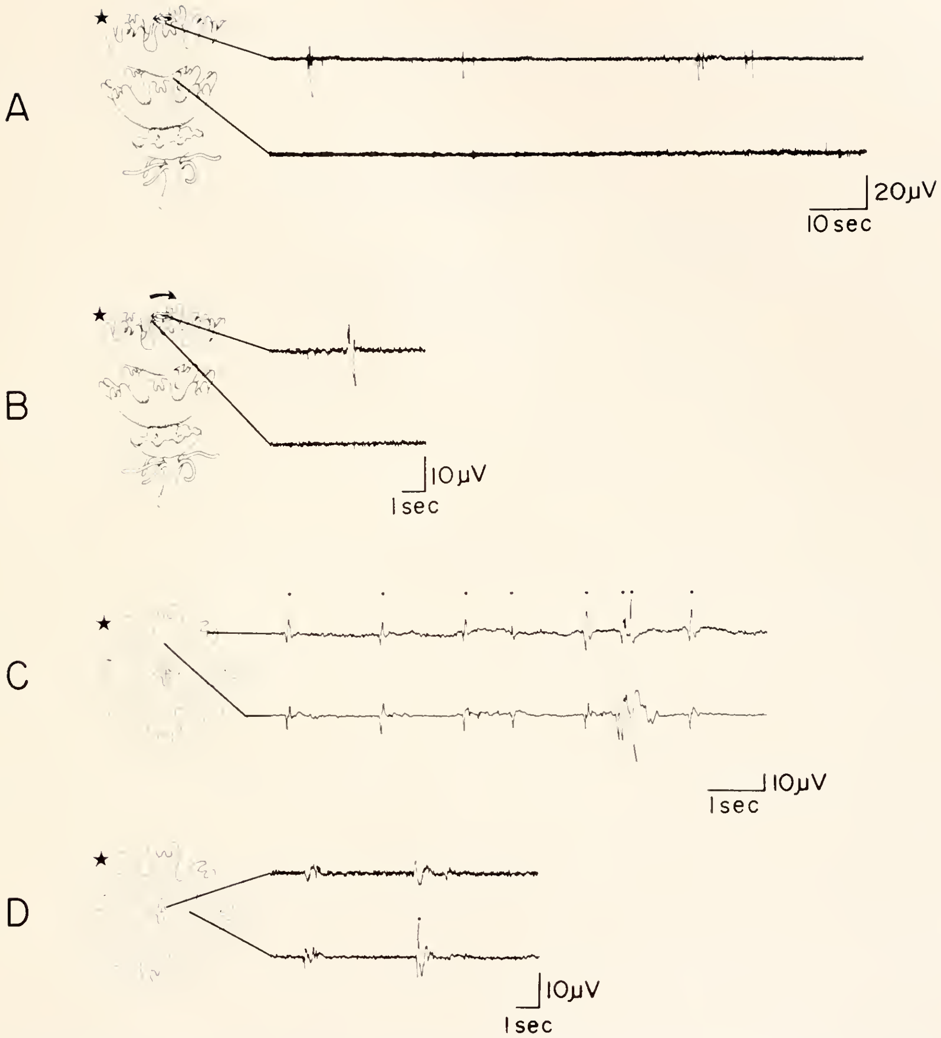

Figure 6. Electrical activity in a strobila. A, records from two manubria, the potentials are not conducted and were not associated with observed behavior; B, potentials recorded from two sides of a manubrium demonstrating the potentials associated with bending (arrow shows the direction of bending); C, top view of a stroblia indicating the electrical activity from a marginal ganglion (upper record) and from a circular muscle in the area indicated; D, top view of a strobila showing the electrical activity in the manubrium and the circular muscles. Dots indicate potentials associated with an observed beating contraction. The star indicates a beating ephyral bud.

recording from two marginal ganglia separated by a third marginal ganglion, shifting pacemaker dominance is obvious. For example, three sets of potentials are shown in Fig. 4D: in the first set the lower MGP occurs after ("a") the upper MGP, in the second set both occur simultaneously ("s"), and in the third set the lower MGP occurs before ("b") the upper MGP.

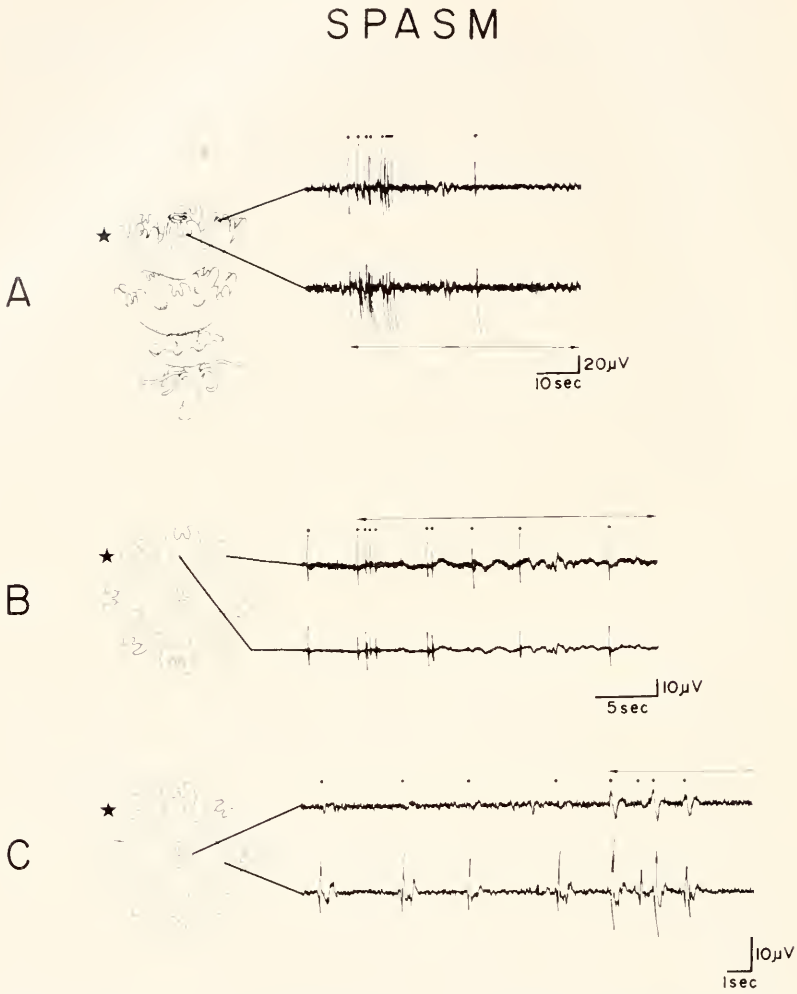

FIGURE 7. Electrical activity during spasm. A, recordings between two marginal ganglia showing the burst; B (top view of a strobila), electrical records between a marginal ganglion (upper record) and a radial muscle (lower record); C, (top view of a strobila) showing records of the electrical activity before a spasm from the manubrium (upper record) and from a radial muscle (lower record). The arrow indicates the duration of the spasm. Dots indicate potentials associated with an observed beating contraction. The star indicates a beating ephyral bud.

Following the appearance of conduction between marginal ganglia, conduction of MGPs could be detected in other parts of the beating ephyra. MGPs were never detected in the exumbrellar surface (Fig. 5A, B) but were routinely recorded from the subumbrellar surface in the areas of the radial and circular musculature (Fig. 5B, Fig. 6C, D). Occasionally, potentials which were associated with the beat occurred in the manubrium but this was unusual (Fig. 6D). MGPs from a beating ephyral bud are not conducted downward to either nonbeating ephyrae or the resting polyp (Fig. 5D). Figure 5D also shows that nonbeating ephyrae do not produce MGPs, and the resting polyp produces only the typical TCPs. Electrical events between ephyrae on the same strobila were independent of each other (Fig. 5C).

Some potentials recorded from the manubrium were not associated with MGPs nor with any observable behavior. These potentials, to use Passano's (1965) terminology, are "cryptic" (Fig. 6A). In addition to MGPs and cryptic potentials, there is also electrical activity which corresponded with the bending of the manubrium (Fig. 6B). During the feeding response in a free ephyra, the manubrium bends toward an infolding arm (Horridge, 1956a). Potentials associated with this bending only occur on the contracting side (Fig. 6B).

Free ephyrae as well as ephyrae still attached to the strobila demonstrate spasm, a maintained oral flexion of the arms (Horridge, 1956a). Apparently, spasm is initiated by a burst of MGPs. Accompanying the burst a series of beats occur which draws all the arms inward (Fig. 7A). When the MGP burst is over, the arms remain folded, and further isolated MGPs occur (Fig. 7A), but during these isolated MGPs only the circular muscles contract. Gradually the radial muscles relax, and the arms are re-extended without any associated electrical activity. Figure 7B shows a complete electrical record of a spasm beginning with an MGP burst and continuing for a time with isolated MGPs. Also shown is the same MGP burst recorded from a radial muscle in another arm. A burst of electrical activity can also be recorded from the manubrium, which is associated with the MGP burst (Fig. 7C), but the isolated MGPs which occur after spasm is established are not reflected by any electrical activity in the manubrium. It should be noted that the experiments depicted in Figure 6D and Figure 7C are recordings from both DNN (in the manubrium) and GFNN (over the radial and circular muscles) in order to demonstrate coupling between the two nets (see Discussion for details).

Spasm in the strobila can be induced by electrical stimulation. Stimulation of the terminal ephyra resulted in spasm of the beating ephyrae in company with the symmetrical shortening of the whole column including the resting polyp. Stimulation of the resting polyp at the same intensity caused no response in the strobila. As stimulus intensity was increased, the excitation spread upwards causing spasm in the proximal, beating ephyra. Upon a further increase in stimulus intensity, spasm was initiated in the whole stack of beating ephyrae.

Discussion

Two types of behavior were observed in the polyp: first, local responses, restricted to one portion of the polyp and independent of activity in other parts of the animal; and, secondly, coordinated behavior involving integrated responses in several body parts. The presence of local responses suggests a single nerve net which is regionally differentiated so that isolated actions can be performed (Chapman, 1965). Previously, when two types of behavior have been observed in the same coelenterate tissue, two conduction pathways have been suggested and sometimes found histologically (Horridge, 1956a). Although double innervation from two nerve-nets cannot be excluded, a combination of epithelial conduction in conjunction with a nerve-net is an equally sound hypothesis. The latter is supported by Chapman's (1965) histological observation of intermuscular junctions. The dimensions of these junctions were not discussed, but they may be low resistance pathways for epithelial conduction as found in many nerve-free coelenterate tissues (Mackie, 1970; Spencer, 1974b; Campbell, Josephson, Schwab, and Rushforth, 1976). Electrically excitable epithelia are relatively insensitive to Mg^{+2} anaesthesia and continue to respond to electrical stimulation after nervous conduction and muscle contractions have been abolished (Mackie and Passano, 1968). The epithelia of medusae or scyphistomae of *A. aurita* treated with excess Mg^{+2} are not electrically excitable; therefore, physiological evidence for epithelial conduction in *A. aurita* is lacking, and the duplicity of behavior in the tentacles remains unexplained.

The polyp does demonstrate two behaviors, feeding and the protective response, which are not regionally independent but involve coordination between body parts and, in the absence of demonstrated epithelial conduction, neuronal conduction is assumed. Feeding is a coordinated action between a tentacle and the mouth. Since the mouth gapes *before* the tentacle reaches the lip, mechanical coordination as suggested by Chapman (1965) is unlikely.

The coordinated feeding and protective response in the scyphistoma are similar to feeding and spasm in the ephyra. When an ephyral arm comes in contact with the prey, the arm moves independently toward the mouth as a radially segmented response. During spasm, the protective response of the ephyra, all the arms are coordinated and bend toward the mouth. Spasm, then, has been interpreted as the feeding response spread to the regionally segmented, independent, sensory nerve-net or diffuse nerve-net (DNN) by interneural facilitation (Horridge, 1956a). The similarity between the behaviors in the scyphistoma and the ephyra suggests that the protective response of the polyp is the feeding response spread to all the tentacles by interneural facilitation. If this assumption is correct, the ephyra retains two polypoid behaviors, feeding and the protective response. The coordination pathway for the protective response in the polyp apparently is in the oral disk since symmetrical column contraction can still take place when polyps are split from the pedal disk to just below the oral disk (Chapman, 1965). A bundle of neurites at the abaxial base of each tentacle has been identified (Chapman, 1965), but whether these bundles form a continuous ring which would provide a coordination pathway for the protective response is not known.

Tentacles and rhopalia (marginal ganglia) are considered to be homologous since rhopalia develop from tentacles and tentacles from rhopalia, as is demonstrated by intermediate teratogenic developmental forms (Thiel, 1966). In addition to the homology suggested by Thiel, certain functional analogies also exist between the tentacles and rhopalia. First, the tentacles seem to be the most sensitive structures of the polyp (Chapman, 1966) so also the rhopalia, since they have been shown to contain light sensitive areas (Horridge, 1959; Yamashita, 1957), respond to gravity (Horridge, 1959), and contain other sensory cells (Horridge, Chapman and MacKay, 1962; Russell, 1970; Chapman and James, 1973). Secondly, the rhopalia of the medusa and the tentacles of the scyphistoma are the most electrically active tissues in these animals. Finally, the electrical activity, the TCP, originates in the distal end of the tentacle and is conducted proximally similar to the conduction direction of the marginal ganglion potential (MGP) through the giant fiber nerve-net (GFNN) to the swimming muscles in the medusa. This evidence indicates that the tentacles and rhopalia are functionally similar structures.

In the early stages of strobilation, when the strobila still possesses its original tentacles, the electrical activity is polypoid. It is interesting that although tentacles and marginal ganglia are probably homologous, there is not a smooth transition between TCPs and MGPs. During strobilation TCPs disappear with the resorption of the tentacles and the ephyral bud is, for a time, electrically quiescent until the occurrence of the first MGPs. In spite of the similarities between tentacles and marginal ganglia, pacemaker activity in the latter may develop *de novo*.

The first characteristic of the adult swimming system to be observed in a strobila was spontaneous beating of individual ephyral arms of the terminal ephyral bud. Potentials associated with the activity corresponded one-to-one with the mechanical activity in that arm. These potentials are MGPs, the same potentials used to drive the swimming muscles in medusae. At first the MGPs are not conducted to other marginal ganglia, but gradually the GFNN becomes functionally established, and the impulses conducted. When conduction is established the arms beat in unison.

In the ephyra, the DNN is distributed over the aboral surface, over the musculature and around the manubrium on the oral surface, while the GFNN is found only in the region of the marginal ganglia and over the radial and circular muscles (Horridge, 1956a). Therefore, the simultaneous recordings of electrical activity in the manubrium and radial musculature during spasm show that the DNN and GFNN become coupled during spasm. In fact, the DNN triggers the MGP burst which initiates spasm. In the medusa a single MGP can be triggered by a single or double DNN pulse after a delay, usually 1.55 sec in *Cassiopea* (Passano, 1973), but such a large delay was not observed in the ephyra. Pacemaker triggering systems are very common in hydrozoan polyps (Passano and McCullough, 1962; Josephson and Mackie, 1965; Morin and Cooke, 1971; Ball and Case, 1973). Furthermore, they are far more complicated than the MGP triggering system described here and are associated with non-nervous epithelial conduction.

The transition of a sessile, phlegmatic polyp to an active, swimming ephyral larva involves the development of a new nerve net, the GFNN, in addition to circular and radial striated muscles. The GFNN, with its pacemakers and through-conducting character, is used to initiate and coordinate swimming. The ephyra, however, does retain the polypoid nerve-net, the DNN, and two polypoid behaviors, feeding and the protective response (spasm) coordinated by the DNN. When ephyrae mature into medusae, the ephyral feeding and spasm responses are lost along with the radial musculature needed for these behaviors. Although feeding and spasm are lost in the medusa, the DNN remains, is sensory in function, controls marginal tentacle contractions, and serves to modulate pacemaker output (Romanes, 1877; Passano, 1965).

The comparison of the behavior and electrophysiology of the polyp, strobila, and medusa of *A. aurita* implies that as development proceeds, each subsequent stage acquires some new behavior while retaining some aspect from the previous stage. While the polypoid character of the DNN is not obvious in the medusa of *A. aurita*, it is clear in other adult scyphozoan medusae which retain the radial musculature. Adult individuals of *Cyanea capillata, Cassiopea andromeda* and

Nausithöe punctata all retain the radial muscles and utilize the DNN to control the asymmetrical compensatory movements used in the righting response (Horridge, 1956b). The compensatory movement in *C. capillata,* for example, occurs by the lengthening of the relaxation of the radial muscles by double innervation (Horridge, 1956b). This lengthening of relaxation is also observed during spasm and feeding in the ephyra of *A. aurita.* In *N. punctata,* feeding behavior is retained in the adult and closely resembles feeding behavior in the ephyra of *A. aurita.* Horridge (1956b) has found that the DNN in *N. punctata* resembles that of the ephyra of *A. aurita* histologically, physiologically, and anatomically. Horridge's observations on adult medusae with radial muscles provide additional evidence that the DNN in medusae is a polypoid structure; however, the behavior subserved by the DNN in the polyp is either lost or modified in the adult medusa. Behaviorally the ephyra is not a medusa. As the ephyra matures into an adult medusa, morphological and behavioral changes occur, not just growth.

SUMMARY

1. Electrical correlates of behavioral activity were observed in the lip and tentacles of the polyp, but none were detected during column contraction. The tentacles are the most electrically active tissue, and the potentials are conducted along the length of the tentacle, but conduction to other parts of the animal were not observed.

2. Although the tentacles of the polyp and the rhopalia of the medusa are probably homologous, the development of pacemaker activity during strobilation is not a smooth transition from tentacle contraction potentials (TCPs) to marginal ganglion potentials (MGPs). This result indicates that each pacemaker activity develops *de novo.*

3. Two types of behavior were observed in the polyp: local responses, and coordinated activity which involved integrated responses in several body parts. The coordinated responses indicate that neurological coordination can take place in the polyp. Furthermore, feeding and spasm in the ephyra are similar to feeding and the protective response in the polyp. This similarity suggests that both coordinated responses in the polyp are coordinated by interneural facilitation in the diffuse nerve net (DNN) as in the ephyra.

4. Swimming in the ephyra is a medusoid behavior but feeding and spasm are coordinated by the DNN and are polypoid responses. Therefore, the ephyra is a mixture of polypoid and medusoid behaviors. As the ephyra matures into an adult medusa both polypoid responses are lost, but the DNN remains to modulate pacemaker output and control marginal tentacle contractions. As development proceeds from polyp, to ephyra, to medusa, each subsequent stage acquires some new behavior while retaining some aspect from the previous stage.

LITERATURE CITED

BALL, E. E., 1973. Electrical activity and behavior in the solitary hydroid *Corymorpha palma.* I. Spontaneous activity in whole animals and in isolated parts. *Biol. Bull.,* **145**: 223–242.

BALL, E. E., AND J. F. CASE, 1973. Electrical activity and behavior in the solitary hydroid *Corymorpha palma.* II. Conducting systems. *Biol. Bull.,* **145**: 243–264.

Bullock, T. H., and G. A. Horridge, 1965. Coelenterata and Ctenophora. Pages 459–534 in *Structure and function in the nervous systems of invertebrates,* Freeman, San Francisco.

Campbell, R. D., R. K. Josephson, W. E. Schwab, and N. B. Rushforth, 1976. Excitability in nerve-free hydra. *Nature,* **262**: 388–390.

Chapman, D. M., 1965. Coordination in a scyphistoma. *Am. Zool.,* **5**: 455–464.

Chapman, D. M., 1966. Evolution of the scyphistoma. *Symp. Zool. Soc. Lond.,* **16**: 51–75.

Chapman, D. M., and R. James, 1973. Intraepithelial flagella in the medusa of *Aurelia aurita* (L). *Publ. Seto Mar. Biol. Lab.,* **20**: 731–743.

Horridge, G. A., 1953. An action potential from the motor nerves of the jellyfish, *Aurellia aurita* Lamark. *Nature,* **171**: 400.

Horridge, G. A., 1954. Observations on the nerve fibres of *Aurellia aurita. Q. J. Microsc. Sci.,* **95**: 85–92.

Horridge, G. A., 1956a. The nervous system of the ephyra larva of *Aurellia aurita. Q. J. Microsc. Sci.,* **97**: 59–75.

Horridge, G. A., 1956b. The nerves and muscles of medusae. V. Double innervation in scyphozoa. *J. Exp. Biol.,* **33**: 366–383.

Horridge, G. A., 1959. The nerves and muscles of medusae. VI. The rhythm. *J. Exp. Biol.,* **36**: 72–91.

Horridge, G. A., D. M. Chapman, and B. MacKay, 1962. Naked axons and symmetrical synapses in an elementary nervous system. *Nature,* **193**: 899–900.

Josephson, R. K., 1965. Three parallel conducting systems in the stalk of a hydroid. *J. Exp. Biol.,* **42**: 139–152.

Josephson, R. K., and G. O. Mackie, 1965. Multiple pacemakers and behavior of the hydroid *Tubularia. J. Exp. Biol.,* **43**: 293–332.

Josephson, R. K., and M. Macklin, 1969. Electrical properties of the body wall of hydra. *J. Gen. Physiol.,* **53**: 638–665.

Mackie, G. O., 1970. Neuroid conduction and the evolution of conducting tissues. *Q. Rev. Biol.,* **45**: 319–332.

Mackie, G. O., and L. M. Passano, 1968. Epithelial conduction in hydromedusae. *J. Gen. Physiol.,* **52**: 600–621.

McFarlane, I. D., 1969. Co-ordination of pedal-disk detachment in the sea anemone *Calliactis parasitica. J. Exp. Biol.,* **51**: 387–396.

Morin, J. G., and I. M. Cooke, 1971. Behavioral physiology of the colonial hydroid *Obelia.* I. Spontaneous movements and correlated electrical activity. *J. Exp. Biol.,* **54**: 689–706.

Passano, L. M., 1965. Pacemakers and activity patterns in medusae: homage to Romanes. *Am. Zool.,* **5**: 465–481.

Passano, L. M., 1973. Behavioral control systems in medusae; a comparison between hydro- and scyhomedusae. *Publ. Seto Mar. Biol. Lab.,* **20**: 615–645.

Passano, L. M., and C. B. McCullough, 1962. The light response and the rhythmic potentials of hydra. *Proc. Natl. Acad. Sci. U.S.A.* **48**: 1376–1382.

Romanes, G. J., 1876. Preliminary observations on the locomotor system of medusae. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.,* **166**: 269–313.

Romanes, G. J., 1877. Further observations on the locomotor system of medusae. *Philos. Trans. R. Soc. Lond. B Biol. Sci.,* **167**: 659–751.

Rushforth, N. B., 1971. Behavioral and electrophysiological studies of hydra. I. Analysis of contraction pulse patterns. *Biol. Bull.,* **140**: 255–273.

Rushforth, N. B., and D. S. Burke, 1971. Behavioral and electrophysiological studies of hydra. II. Pacemaker activity of isolated tentacles. *Biol. Bull.,* **140**: 502–519.

Russell, F. S., 1970. *The Medusae of the British Isles.* II. *Pelagic scyphozoa, with a supplement to the first volume on hydromedusae.* Cambridge University Press, Cambridge, 284 pp.

Schäfer, E. A., 1879. Observations on the nervous system of *Aurelia aurita. Philos. Trans. R. Soc. Lond. B Biol. Sci.,* **169**: 563–575.

Spangenberg, D. B., 1971. Thyroxine induced metamorphosis in *Aurelia aurita. J. Exp. Zool.,* **178**: 183–194.

Spencer, A. N., 1974a. Behavior and electrical activity in the hydrozoan *Proboscidactyla flavicirrata* (Brandt). I. The hydroid colony. *Biol. Bull.,* **145**: 100–115.

SPENCER, A. N., 1974b. Non-nervous conduction in invertebrates and embryos. *Am. Zool.,* **14**: 917–929.

STOKES, D. R., 1974. Physiological studies of conducting systems in the colonial hydroid, *Hydractinia echinata. J. Exp. Zool.,* **190**: 1–18.

THIEL, H., 1966. The evolution of the scyphozoa. A review. *Symp. Zool. Soc. Lond.,* **16**: 76–117.

YAMASHITA, T., 1957. Das Actionpotential der Sinneskorper (Randkorper) der Meduse *Aurelia aurita. Z. Biol.,* **109**: 116–122.

Reference: *Biol. Bull.*, 152: 251–262. (April, 1977)

THE ONTOGENY OF SWIMMING BEHAVIOR IN THE SCYPHOZOAN, *AURELIA AURITA*. II. THE EFFECTS OF IONS AND DRUGS[1]

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The active swimming medusa of the scyphozoan, *Aurelia aurita,* develops from a rather inactive, sessile polyp, the scyphistoma. Seasonally, larval medusae (ephyrae) develop by transverse budding of the scyphistoma. This process of medusa formation is termed strobilation, and the scyphistoma at this stage is called a strobila. The ephyrae begin swimming movements while still attached to the strobila. After swimming activity begins, the ephyrae are released to grow and mature into medusae.

Prior to the production of medusae the musculature of a scyphistoma is entirely nonstriated and the nervous system consists of a diffuse nerve net (DNN) functionally arranged in segments (Chapman, 1965, 1966). The development of swimming behavior involves the acquisition of the striated swimming muscles, ganglionic pacemakers, and a through-conducting nerve net which coordinates the swimming beats. The sequence of behavioral and electrophysiological events occurring during the ontogeny of swimming activity are described in the preceding paper (Schwab, 1977), with the conclusion that the coordinating mechanisms involved in swimming are new features of the medusa and not simply modifications of polyp mechanisms. This paper is an investigation undertaken to test this hypothesis by examining the effects of ions and the drugs on the behavior of the scyphistoma, strobila, and adult medusa.

There have been a number of studies examining the effects of ionic variation on the swimming rhythm of scyphomedusae. The effects of the major ions in sea water were determined by using solutions containing those ions in excess of sea water concentration (Mayer, 1906, 1914; Bullock, 1943; Horridge, 1959). The results obtained by these investigators are difficult to interpret since osmotic concentration was not maintained constant. In this study the major ions were reduced or deleted and the osmotic concentration kept constant by substituting another ionic species for the deleted ion.

There have also been many studies on the effects of pharmacological agents on the swimming rhythm of scyphomedusae. Romanes (1877, 1885) observed the effect of various "poisons" (chloroform, "strichnia," curare, and "morphia") on scyphomedusae and found them to have inhibitory effects on the swimming rhythm. Horridge (1959) found that tryptamine accelerates the swimming rhythm of *Cyanea* and *Aurelia* but acetylcholine with and without physostigmine, adrenaline,

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curare, ephedrine, histamine, and 5-hydroxytryptamine had no effect. A substance, as yet unidentified, has been extracted from marginal ganglia of *A. aurita* and found to either increase or decrease spontaneous swimming activity of *A. aurita* depending on the concentration (Barnes and Horridge, 1965). Scyphomedusae, and probably coelenterates as a group, are pharmacologically different from other phyla in being generally unresponsive to common neurohumors (Horridge, 1959; Barnes and Horridge, 1965) and to tetrodotoxin (Mackie, 1968; Schwab, 1972; Ball and Case, 1973).

The approach used in this study is to examine the effects of a number of pharmacological agents and ionic alterations on the behavior of medusae and compare those responses with those of the scyphistoma and strobila. This approach is based on the idea that different coordinating systems in the several life stages might be reflected as different response patterns to ions and drugs. With the response patterns for the medusa established and divided into classes, one or two of the test solutions were selected from each response class and tested on the scyphistoma and strobila. Finding different response patterns would strengthen the hypothesis that coordinating systems of the different life stages are fundamentally different and not simply subtle alterations of the elements present in previous stages.

MATERIALS AND METHODS

Animals

Medusae of *Aurelia aurita* (5–8 cm in diameter) were collected from the Eel Pond, Woods Hole, Massachusetts. The medusae were kept in a deep tank with slowly flowing sea water until they were used. Polyps of *A. aurita* were obtained from the Supply Department, Marine Biological Laboratory, Woods Hole, Massachusetts. Some additional polyps (Woods Hole strain) were obtained from Dr. Dorothy Spangenberg (University of Colorado, Boulder, Colorado). Scyphistomae were maintained in I^--free artificial sea water (ASW) to prevent strobilation (Spangenberg, 1971; for additional details on the maintenance of polyp cultures see Schwab, 1977). Scyphistomae, previously maintained in I-free ASW, strobilate in 6–8 weeks after the polyps are transferred to ASW containing 10^{-5} M KI (Spangenberg, personal communication).

Recording methods

The mechanical activity of swimming medusae was recorded from animals in a constant-temperature chamber containing either sea water (SW) or a test solution maintained at the storage tank temperature (15–18° C). The animals were individually suspended by a hook placed in the aboral mesoglea and connected by silk suture *via* a light spring to a force-displacement transducer. Raising or lowering the transducer adjusted the tension so that the medusa was kept off the bottom of the chamber. The output of each transducer was recorded on a four-channel oscillograph. In order to reduce artifacts resulting from swimming movements, electrical recordings were made from isolated marginal ganglia. Marginal ganglia were removed from medusae and pinned, aboral side up, in a constant temperature chamber adjusted to the temperature of the SW holding tank. A glass suction electrode with a tip aperture of 50 μm (Schwab, 1977) was attached to the aboral surface of the rhopalium near the ocellus.

Mechanical activity of the tentacles of the scyphistoma was monitored visually or by a photoelectric device and electrical activity was detected with glass suction electrodes (Schwab, 1977). Mechanical activity of the strobila was recorded by observing the tissue and manually deflecting an event marker on a penwriter. Electrical activity from the marginal ganglia of attached ephyrae was recorded by glass suction electrodes.

Electrical potentials from medusae, ephyrae, and scyphistomae were recorded between the glass suction electrode and an Ag/AgCl, indifferent bath electrode. Recorded activity was amplified by a high gain AC-amplifier with a long-time constant and displayed on an oscilloscope and penwriter.

Test solutions

Artificial sea water (ASW) and isosmotic ASW test solutions of various ionic compositions were made from stock solutions prepared with reagent grade chemicals and deionized distilled water (Wilkens, 1970). Cl^--free solutions were prepared with either isethionate or propionate as chloride substitutes. The Cl^--free isethionate solution contained 491.4 mM Na^+, 10.0 mM K^+, 9.8 mM Ca^{+2}, 50.8 mM Mg^{+2}, 65.6 mM SO_4^{-2}, and 491.4 mM isethionate. The concentrations of the ionic species in all other isomotic test solutions are tabulated in Wilkens (1970).

Isosmotic test solutions containing an ionic concentration less than that found in SW were prepared by mixing SW with the ion-free solution in appropriate volumes. The ionic concentration of these test solutions will be expressed as a percentage of the concentration of that ionic species normally found in SW. The test solution containing 108% Na^+ was a 1 : 1 mixture of sea water and 0.54 M NaCl (*i.e.*, 50% Ca^{+2}, 50% K^+, and 50% SO_4^{-2}). The solutions containing pharmacological agents were prepared by dissolving the chemical in SW. The pH and osmotic concentration of all test solutions were determined before use. The solutions were titrated with either NaOH or HCl to pH 7.8 (pH of SW). Osmotic concentrations were measured with a freezing point depression osmometer and adjusted to 925 mOsmol with deionized distilled water.

Experimental design

The protocol used for recording mechanical swimming activity of medusae consisted of a 20 min SW control period, a 20 min test period, and a 20 min SW recovery period. At the end of the control period the SW was removed from the chamber and replaced with 200 ml of the test solution. Following the test period, medusae which ceased to swim were electrically stimulated with platinum pin electrodes, insulated to the tip and inserted into the mesoglea immediately adjacent to the circular swimming muscles. The animals were electrically stimulated once per sec (150 V, 10 msec) for 10 to 15 sec. This gross stimulation determined if the swimming muscles were capable of contracting in the test solution. Following this stimulation, the test solution was drawn off, the chamber and animals washed with 50 ml SW and, finally, the test solution replaced by 200 ml SW.

The mechanical activity in each test solution was analyzed by counting the beats per min (bpm) for each animal during the 20 min control period and the last 5 min of the test period, determining the difference between them and obtaining the mean difference for the experimental group. The last 5 min of the test period was used in order to obtain the maximum effect of the test solution in the test period. The same control, test, and recovery periods, as well as the solution exchanging procedure used during the mechanical recordings of intact medusae, were also used with isolated ganglia.

Since the tentacles of the polyp are developmentally homologous to the rhopalia of medusae (Thiel, 1966) and are the most electrically active tissue of the polyp (Schwab, 1977), this study was restricted to the differential responses of the tentacles to ions and pharmacological agents.

The same 20 min control period, test period, and recovery period used with medusae was also used with scyphistomae. Electrical and mechanical recordings, however, were done simultaneously rather than sequentially as with medusae. To test the responsiveness of the muscles in the presence of the test solution, the tentacles were electrically stimulated *via* platinum pin electrodes insulated to the tip with teflon. Solutions were added to the test container (see Schwab, 1977) by a gravity fed, polyethylene tube system. As the solution flowed into the test container it overflowed into a larger, outer chamber from whence it was withdrawn by a vacuum line, thus maintaining constant the water level of the test chamber. Fifty ml of a new solution was used to wash out the 1.4 ml volume of the test container. Preliminary experiments had determined that a 10 ml/min rate of flow through the test chamber did not result in any observable response of the polyp to the mechanical stimulus of the changing solution.

The effects of ionic variation and drugs on the electrical and mechanical activity associated with beating activity of ephyrae attached to a strobila were determined by using the same 20 min control, test and recovery period. The methods of recording electrical and mechanical activity, electrical stimulation, and solution exchanging procedure were the same as that used with scyphistomae. All experiments on both scyphistomae and strobilae were done at 4° C in a constant-temperature bath to maintain the animals at the temperature in which they were cultured.

RESULTS

Effects of test solutions on the swimming system of medusae

The effects of test solutions on the mechanical events of swimming are summarized in Table I. Total inhibition of swimming activity was defined as the complete absence of spontaneous swimming activity in all animals tested during the last 5 min in the test solution. Swimming muscles were considered nonfunctional when they did not respond to electrical stimulation during total inhibition of swimming activity. The effects of all solutions tested fell into four types (Table I).

Type I showed no effect on muscles or marginal ganglia. Typical records of the Type I response are shown in Figure 1.

Type II showed total or partial inhibition of swimming beats and marginal ganglion activity, but animals were still responsive to electrical stimulation (Fig. 1). Ten test solutions had a Type II effect on medusae (Table I) and apparently resulted from inhibition of marginal ganglion activity. Intermediate Na^+ concen-

TABLE I

Effects of test solutions on the mechanical activity of the swimming muscles and electrical activity of the marginal ganglia.

Response type	Test solution (isosmotic test solutions from Wilkins, 1970)	Mean difference in beat frequency (beats/min ± s.e.; +, increase; −, decrease in frequency)	N	Total inhibition of spontaneous swimming activity?	Inhibition of MGPs?	Muscle responds to electrical stimulation? (— = not tested)
I	ASW	− 5.3 ± 5.1	12	no	no	—
I	EDTA (2 mM)	+ 2.7 ± 4.0	4	no	no	—
I	EGTA (2 mM)	+ 7.0 ± 4.0	4	no	no	—
I	108% NaCl	−10.8 ± 6.7	4	no	no	—
I	Tyramine (10^{-2} M)	− 3.3 ± 3.6	4	no	no	—
I	Cl^--free (Prop.) ASW	+ 3.0 ± 4.5	6	no	no	—
II	Na^+-free ASW	−31.9 ± 3.6*	6	yes	yes	yes
II	Li ASW	−33.9 ± 2.5*	6	no	yes	—
II	K^+-free ASW	−25.1 ± 1.3*	6	no	yes	—
II	Cl^--free (ISE) ASW	−20.2 ± 3.8*	4	no	yes	—
II	Ca^{+2}-free ASW (+EGTA)	−27.4 ± 3.3*	7	no	yes	—
II	Ca^{+2}-free ASW (NC)**	−38.8 ± 3.4*	8	no	yes	—
II	Ca^{+2}-free Li ASW	−37.6 ± 4.3*	4	no	yes	—
II	Na^+-Ca^{+2}-free ASW (+EGTA)	−27.6 ± 4.2*	4	no	yes	—
II	Na^+-Ca^{+2}-free ASW (NC)**	−36.5 ± 2.6*	4	no	yes	—
II	Na^+-Ca^{+2}-Mg^{+2}-free ASW (+EDTA)	−34.5 ± 5.0*	4	yes	yes	yes
III	Procaine (10^{-2} M)	−33.1 ± 3.2*	6	yes	yes	no
III	Caffeine (10^{-2} M)	−38.2 ± 3.7*	6	yes	yes	no
III	Tryptamine (10^{-2} M)	−30.0 ± 3.8*	4	yes	yes	no
III	Veratrine (1:10^3 w/v)	−26.7 ± 0.9*	4	yes	yes	no
IV	Mg^{+2}-free ASW (+EDTA)	+30.5 ± 5.6*	4	no	no	—
IV	Mg^{+2}-free ASW (NC)**	+20.5 ± 4.0*	4	no	no	—
IV	Ca^{+2}-Mg^{+2}-free ASW (+EDTA)	+14.3 ± 2.7*	4	no	no	—
IV	Ca^{+2}-Mg^{+2}-free ASW (NC)**	+18.7 ± 2.8*	4	no	no	—

* $P < 0.05$.
** NC = No chelator.

trations, between 0% Na^+, and 50% Na^+ concentrations, resulted in significant decreases in the rate of swimming activity (Table II).

Type III showed inhibition of swimming beats and MGPs, and the preparation was unresponsive to electrical stimulation (Fig. 1). The effective doses for these compounds are shown in Table III. Characteristically the effect of these drugs, especially at the lower concentrations, was a decrease in contraction amplitude without a change in rate. Similarly, recovery was characterized by an increase in contraction height without a change in rate. In tryptamine (10^{-2} M) or veratrine (1 : 10^3 w/v), medusae failed to recover during the 20 min recovery period; how-

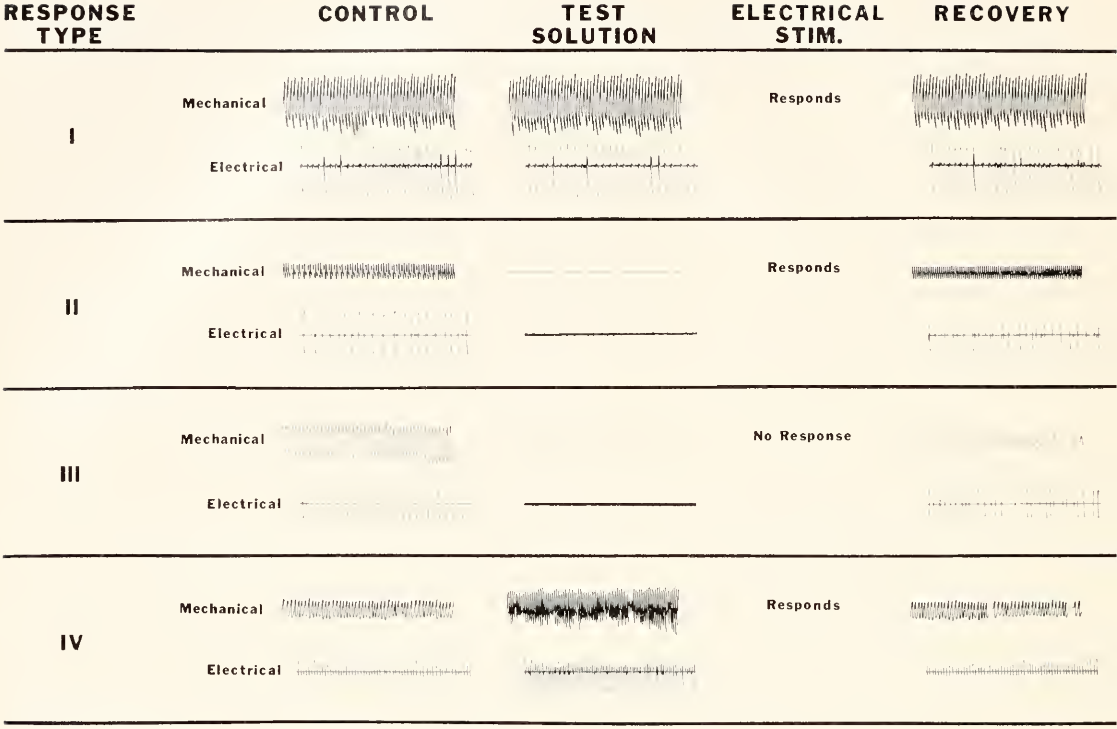

FIGURE 1. Sample records of the mechanical responses of intact medusae (upper traces) and electrical responses of isolated marginal ganglia (lower traces) to test solutions classified according to response types: I, no effect; II, inhibitory effect on pacemaker activity; III, both pacemaker activity and swimming muscles were inhibited; IV, pacemakers excited.

ever, these drugs could be washed out after an extended period in SW. In the case of veratrine, for instance, small contractions began 3 hr after SW replaced the test solution.

TABLE II

Effects of reduced ionic concentrations on the swimming system of A. aurita.

Response type	Test solution (% of SW)	Mean difference in beat frequency (beats/min ± s.e.; +, increase; −, decrease in frequency)	N	Total inhibition of spontaneous swimming activity	Inhibition of MGPs?	Muscle responds to electrical stimulation? (— = not tested)
II	50% Na^+	−39.9 ± 2.5*	4	no	yes	—
II	10% Na^+	−36.7 ± 4.7**	4	yes	yes	yes
II	10% K^+	−26.6 ± 3.6**	4	no	yes	—
II	5% Ca^{+2}	−19.4 ± 4.0*	4	no	yes	—
IV	20% Mg^{+2}	+22.4 ± 3.5*	4	no	no	—
IV	10% Mg_{+}^{2}	+22.9 ± 4.2*	3	no	no	—

* $P < 0.05$.
** $P < 0.01$.

Table III

Effects of drugs on the swimming system of A. aurita.

Response type	Test solution	Mean difference in beat frequency (beats/min ± s.e.; +, increase; −, decrease in frequency)	N	Total inhibition of spontaneous swimming activity?	Inhibition of MGPs?	Muscle responds to electrical stimulation? (— = not tested)
III	10^{-2} M Procaine	-33.1 ± 3.2*	6	yes	yes	yes
III	5×10^{-3} M Procaine	-31.7 ± 2.7*	6	yes	yes	yes
II	10^{-3} M Procaine	-24.2 ± 3.9*	6	no	yes	—
II	5×10^{-4} M Procaine	-9.1 ± 3.2*	4	no	yes	—
III	10^{-2} M Caffeine	-38.2 ± 3.7*	6	yes	yes	yes
III	5×10^{-3} M Caffeine	-32.1 ± 2.5*	6	yes	yes	yes
II	2.5×10^{-3} M Caffeine	-19.7 ± 3.1*	6	no	yes	—
II	10^{-3} M Caffeine	-7.3 ± 2.4*	6	no	yes	—
III	10^{-2} M Tryptamine	-25.9 ± 3.8**	4	yes	yes	yes
II	10^{-3} M Tryptamine	-15.2 ± 1.9**	4	no	yes	—
III	$1:10^3$ Veratrine	-26.7 ± 0.9***	4	yes	yes	yes
II	$1:10^4$ Veratrine	-27.4 ± 4.9***	4	no	yes	—

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

Type IV showed an increased rate of mechanical activity. This was seen in solutions lacking Mg^{+2} or with reduced Mg^{+2} concentrations (Table II). In SW the appearance of the bell alternates between a disk (relaxed) and a bell (swimming contraction). During high frequencies of contraction, as in Mg^{+2}-free ASW, activity was erratic and the animal assumed a constant bell shape due to incomplete relaxations (Fig. 1, Type IV). Sometimes this response was more pronounced and the medusa lost coordination; many contractile events could be seen occurring independently in different sectors of the bell. The mechanical activity returned to normal following replacement of the test solution with SW (Fig. 1, Type IV). Similarly, isolated ganglia showed a high rate of activity in both Mg^{+2}-free and Ca^{+2}-free ASW (Fig. 1, Type IV). The increased swimming activity in Ca^{+2}-Mg^{+2}-free ASW is surprising, since reducing Ca^{+2} alone is inhibitory. Apparently the absence of Ca^{+2} cannot reverse the excitatory effect in the absence of Mg^{+2}.

One possible response pattern, inhibition of swimming activity without affecting MGP activity (*i.e.*, uncoupling muscle contractions from GFNN activity), was not seen with any of the test solutions.

Effect of test solutions on the tentacular system of polyps

The tentacular system responded quite differently than the swimming system to the solutions tested (Table IV). Procaine, which inhibits both pacemaker activity and the swimming muscles of medusae and Mg^{+2}-free ASW, which leads to hyperexcitability in medusae, had no effect on spontaneous electrical activity and tentacle movements in polyps. In medusae, Na^{+}-free ASW inhibits marginal pacemaker output in the swimming system but in the polyp both TCPs and tentacle contractility are inhibited. The swimming system and the tentacular system responded similarly

Table IV

Differential responses of the three stages of the life cycle of Aurelia aurita *to test solutions.*

	Response type			
	I (No response)	II (Inhibition of pacemaker activity)	III (Inhibition of pacemakers and swimming muscles)	IV (Increase in pacemaker output)
Swimming system of the medusa	ASW	Na^+-free ASW Ca^{+2}-free Li ASW	Procaine (10^{-2} M)	Mg^{+2}-free ASW
Tentacular system of the polyp	ASW Procaine (10^{-2} M) Mg^{+2}-free ASW	Ca^{+2}-free Li ASW	Na^+-free ASW	
Beating system of the strobila	ASW Procaine (10^{-2} M) Mg^{+2}-free Li ASW		Na^+-free ASW	Mg^{+2}-free ASW

to Ca^{+2}-free ASW. Typical responses of the tentacular system to the test solutions are shown in Figure 2.

Effects of test solutions on the beating system of attached ephyrae

The effects of the test solutions on the beating system are shown in Figure 3. ASW, procaine (10^{-2} M), and Ca^{+2}-free Li ASW had no effect on the beating

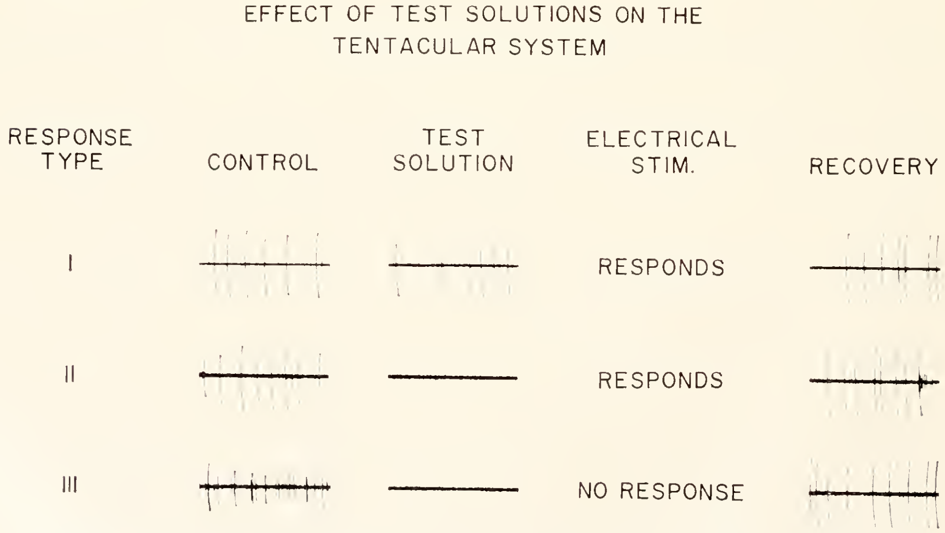

Figure 2. Effect of test solutions on the tentacular system classified according to the response types established for the medusa: I, no effect; II, only the TCPs were inhibited; III, both TCPs and the muscles were inhibited.

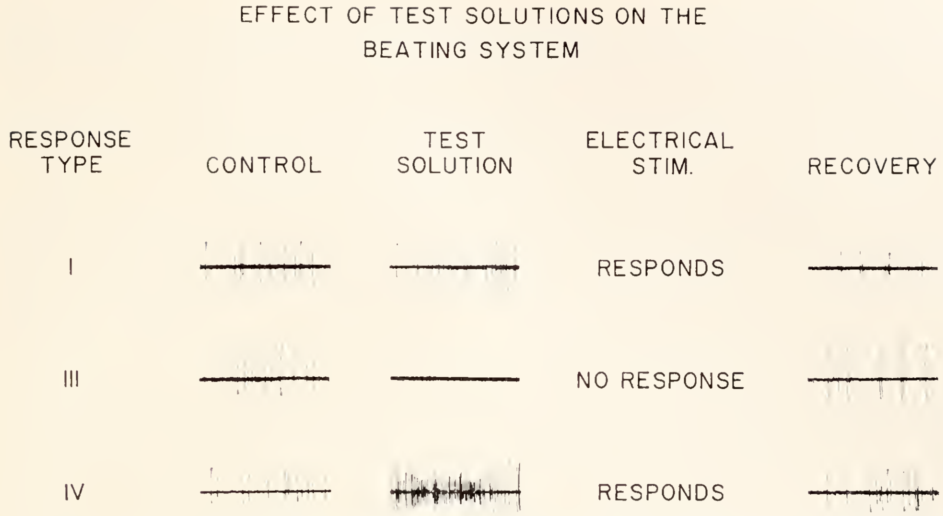

FIGURE 3. Effect of test solutions on the beating system classified according to the response types established for the medusa: I, no effect; III, both pacemakers and beating muscles were inhibited; IV, pacemakers excited.

system. Na^+-free ASW inhibited both electrical and beating activity while Mg^{+2}-free ASW caused an increase in the rate of beating (Table IV).

DISCUSSION

Most of the test solutions had either no effect (Type I) or an inhibitory effect on swimming activity of medusae which resulted from an inhibition of pacemaker activity (Type II) or an inhibition of both pacemakers and swimming muscles (Type III). Four test solutions increased swimming activity (Type IV response). This increased rate of swimming in the absence of Mg^{+2} was surprising, although adding excess Mg^{+2} to sea water is a commonly used anaesthetic for marine invertebrates. *Haliclystus auricula,* a sessile scyphozoan, does not exhibit the swimming contractions normally associated with medusae. Stauromedusae, such as *H. auricula* have been considered to be derived from scyphistomae which failed to completely develop into free medusae (Hyman, 1940). Therefore, residual pacemaker activity has been suspected, but none has been demonstrated (Gwilliam, 1960). Residual pacemaker activity might be detected in the absence of Mg^{+2}; however, *H. auricula* failed to show any excitatory effects thus providing additional evidence for the complete lack of pacemaker activity in these animals. In addition, the hydromedusae, *Sarsia tubulosa* and *Aequorea aequorea,* do not respond with an increase in swimming activity in the absence of Mg^{+2} (Schwab, unpublished observations).

The increased rate of swimming activity in Mg^{+2}-free ASW was caused by an increase in the rate of pacemaker firing. The absolute refractory period of the swimming muscles in sea water is approximately 0.7 sec (Bullock, 1943; Pantin and Vianna Dias, 1952), which corresponds to a maximum possible rate of

85 bpm. The highest stimulation frequency to which a ganglion-free muscle preparation will respond in a one-to-one fashion is 1.3 pulses/sec (Bullock, 1943) or 78 bpm. The rate of activity caused by the test solutions did not exceed the upper limits determined from the control animals (approx 80 bpm). Therefore, the increase in rate probably resulted from pacemaker excitation only, and a decrease in the refractory period of the muscles need not be postulated.

Many investigators have subjected coelenterates to media containing various pharmacologically active compounds. For instance, the sympathomimetics, tryptamine and tyramine, were found to initiate contractions in the anemone, *Calliactis*, but adrenaline was without effect (Ross, 1945, 1957). In the hydroid, *Corymorpha*, tyramine increases electrical activity (Ball and Case, 1973). The medusae of *A. aurita* responded differently to both of these compounds. Tyramine was totally without effect on the swimming system of *A. aurita*, yet tryptamine inhibited both pacemakers and swimming muscles (*contra* Horridge, 1959). Veratrine, a mixture of alkaloids, causes contracture in vertebrate skeletal muscle and spontaneous contractions in *Calliactis* (Ross, 1945) but, at the same concentration, did not cause contracture of the swimming muscles; rather it inhibited both pacemakers and swimming muscles. Caffeine, which also causes contracture in vertebrate skeletal muscle, stimulates vertebrate cardiac muscle and excites the central nervous system, had none of these effects on the medusa of *A. aurita* but rather caused the same inhibition as tryptamine, veratrine, and procaine. Tetrodotoxin (TTX) and procaine both block the action potential in squid axons (Nakamura, Nakajima, and Grundfest, 1965; Taylor, 1959). TTX had no effect on the swimming system (Schwab, 1972) but procaine, previously thought to affect only the swimming muscles (Schwab, 1972), blocks both the swimming muscles and the MGPs. Similarly, TTX has also failed to block electrical activity in the hydroids *Corymorpha* (Ball and Case, 1973) and *Cordylophora* (Mackie, 1968). Many other compounds, effective in other systems (*e.g.*, acetylcholine, adrenaline, histamine, curare, ephedrine, and 5-hydroxytryptamine) are also without effect on the scyphozoan swimming system (Horridge, 1959). This evidence shows that the coelenterate neuromuscular system is pharmacologically quite different from those in other phyla, and further, that the scyphozoan neuromuscular system is pharmacologically quite different from other classes of Cnidaria.

There are several differences between the effects of ionic variation on the swimming activity of *A. aurita* and those reported earlier for other species of medusae (Mayer, 1906; Horridge, 1956a, b). For instance, excess Ca^{+2} totally inhibited swimming activity in *Cassiopea andromeda* and *C. xamanchana* but only partially inhibited swimming activity in *A. aurita*. Excess Na^{+} totally inhibited swimming activity of *C. xamanchana*, whereas excess Na^{+} had no significant effect on the activity of *A. aurita*. Since no information is available on the ionic mechanisms underlying cnidarian nervous activity, these differences, at the moment, cannot be explained. The results also cannot eliminate the possibility that the test solutions which inhibited or stimulated pacemaker output may have affected the inputs to pacemakers rather than pacemakers directly. The exact site of each effect and the mechanism behind the effects remains unknown.

Regardless of mechanism, the responses obtained from the medusa of *A. aurita* are useful as an index of physiological maturity when compared with the responses obtained from other stages of the life cycle. For instance, Mg^{+2}-free ASW, which greatly increased swimming activity in medusae and beating activity in ephyrae, had no effect on the polyp. The excitatory effect of Mg^{+2}-free ASW on the ephyra supports the hypothesis that the excitatory effect of Mg^{+2}-free ASW is general to the pacemakers found in swimming scyphozoan forms. Thus the development of the Type IV response may be considered as the development of a medusoid response. The Type III response to procaine was specific to the adult medusa and is also considered a medusoid response. Physiological development is not complete in the ephyra, since the Type III response to procaine was not observed. Although the comparison of the responses between the polyp, strobila, and medusa show no other clear relationships, the limited number of test solutions used was sufficient to establish that the tentacular system, beating system, and swimming system are physiologically different.

In summary, the transformation of the sessile polyp to an active swimming medusa involves the development of both active pacemakers interconnected by a fast, through-conducting nerve net and striated swimming muscles. Both develop during strobilation causing the ephyra to exhibit swimming behavior similar to the medusa. Perhaps this similarity between the medusa and ephyra previously obscured the similarities between the polyp and ephyra. Now there is evidence that the newly acquired medusoid nerve net and accompanying behavior in the strobila (*i.e.*, ephyra) is superimposed over a polypoid nerve net and behavior (Schwab, 1977). The evidence presented here suggests that the strobila is not only a developmental mixture of both polypoid and medusoid behavioral characteristics but also physiological characteristics. In spite of obvious similarities, the beating system of the ephyra and the swimming system of the medusa are not physiologically or behaviorally identical. Therefore, as the ephyra matures into an adult, morphological maturation must, perforce, be accompanied by further physiological maturation of the neuromuscular system responsible for producing the swimming movements.

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SUMMARY

1. The responses of *Aurelia* medusae to pharmacological agents and ionic variation were classified into four response types: Type I, no response; Type II, inhibition of pacemaker activity; Type III, inhibition of both pacemakers and swimming muscles; and Type IV, increase in pacemaker output.

2. The swimming pacemakers of *Aurelia* medusae become hyperactive in Mg^{+2}-free solutions (Type IV). This response appears to be general in swimming scyphozoa.

3. The response pattern to pharmacologically-active compounds indicates that the coelenterate neuromuscular system is quite different than those in other phyla. In fact, the response spectrum is not consistent within the Cnidaria.

4. Similarly, the responses of adult medusae to ionic variation show no consistent pattern within various scyphomedusae.

5. Test solutions from each response type established with medusae were selected and tested on the scyphistoma and strobila stages. The comparison of

the responses to the test solutions between the medusa, scyphistoma, and strobila showed that the neuromuscular systems are physiologically different. The strobila, specifically the ephyra, is a mixture of both polypoid and medusoid response types. The strobila, therefore, is physiologically an intermediate stage in the development of the adult medusa.

LITERATURE CITED

BALL, E. E., AND J. F. CASE, 1973. Electrical activity and behavior in a solitary hydroid *Corymorpha palma*. II. Conducting systems. *Biol. Bull.,* **145**: 243–264.

BARNES, W. J. P., AND G. A. HORRIDGE, 1965. A neuropharmacologically active substance from jellyfish ganglia. *J. Exp. Biol.,* **42**: 257–267.

BULLOCK, T. H., 1943. Neuromuscular facilitation in scyphomedusae. *J. Cell. Comp. Physiol.,* **22**: 251–272.

CHAPMAN, D. M., 1965. Coordination in a scyphistoma. *Am. Zool.,* **5**: 455–564.

CHAPMAN, D. M., 1966. Evolution of the scyphistoma. *Symp. Zool. Soc. Lond.,* **16**: 51–75.

GWILLIAM, G. F., 1960. Neuromuscular physiology of a sessile scyphozoan. *Biol. Bull.,* **119**: 454–473.

HORRIDGE, G. A., 1956a. The nervous system of the ephyra larva of *Aurellia aurita*. *Q. J. Microsc. Sci.,* **97**: 59–75.

HORRIDGE, G. A., 1956b. The nerves and muscles of medusae. V. Double innervation in scyphozoa. *J. Exp. Biol.,* **33**: 366–383.

HORRIDGE, G. A., 1959. The nerves and muscles of medusae. VI. The rhythm. *J. Exp. Biol.,* **36**: 72–91.

HYMAN, L. H., 1940. Observations and experiments on the physiology of medusae. *Biol. Bull.,* **79**: 282–296.

MACKIE, G. O., 1968. Electrical activity in the hydroid *Cordylophora*. *J. Exp. Biol.,* **49**: 387–400.

MAYER, A. G., 1906. Rhythmical pulsations in animals. I. Pulsations of jellyfishes, arms of *Lepas,* heart of *Salpa* and of loggerhead turtle. *Carnegie Inst. Wash. Publ.,* **47**: 1–62.

MAYER, A. G., 1914. The relation between the degree of concentration of the electrolytes of sea-water and the rate of nerve-conduction in *Cassiopea*. *Pap. Tortugas Lab.,* **6**: 25–54 (*Carnegie Inst. Wash. Publ.,* 183).

NAKAMURA, Y., S. NAKAJIMA, AND H. GRUNDFEST, 1965. The action of tetrodotoxin on electrogenic components of squid axons. *J. Gen. Physiol.,* **48**: 985–996.

PANTIN, C. F. A., AND M. VIANNA DIAS, 1952. Rhythm and after discharge in medusae. *An. Acad. Bras. Cienc.,* **24**: 335–364.

ROMANES, G. J., 1877. Further observations on the locomotor system of *Aurelia aurita*. *Philos. Trans. R. Soc. Lond. B Biol. Sci.,* **169**: 563–575.

ROMANES, G. J., 1885. *Jellyfish, starfish and sea urchins, being a research on primitive nervous systems.* International Science Series D, Appleton and Co., New York, 323 pp.

ROSS, D. M., 1945. Facilitation in sea anemones. I. The action of drugs. *J. Exp. Biol.,* **22**: 21–31.

ROSS, D. M., 1957. The action of tryptamine and 5-hydroxytryptamine on the muscles of sea anemones. *Experientia,* **13**: 192–194.

SCHWAB, W. E., 1972. Some effects of ionic variation on the swimming rhythm of *Aurelia aurita*. *Am. Zool.,* **12**: 693.

SCHWAB, W. E., 1977. The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita*. I. Electrophysiological analysis. *Biol. Bull.,* **152**: 233–250.

SPANGENBURG, D. B., 1971. Thyroxine induced metamorphosis in *Aurelia aurita*. *J. Exp. Biol.,* **178**: 183–194.

TAYLOR, R. E., 1959. Effect of procaine on electrical properties of squid axon membrane. *J. Am. Physiol.,* **196**: 1071–1078.

THIEL, H., 1966. The evolution of the scyphistoma a review. *Symp. Zool. Soc. Lond.,* **16**: 51–117.

WILKENS, L. A., 1970. Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus demissus*. *Ph.D. Thesis, Florida State University,* Tallahassee, Florida, 128 pp. (*Diss. Abstr.,* **31**: 6867; order no. 71–13, 521.)

Reference: *Biol. Bull.*, **152**: 263-274. (April, 1977)

LIMB REGENERATION IN FIDDLER CRABS: SPECIES DIFFERENCES AND EFFECTS OF METHYLMERCURY

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Brachyuran crabs can autotomize limbs at a pre-formed plane in the basi-ischium and subsequently regenerate them. The limbs grow out in a folded position within a layer of cuticle, and at ecdysis they unfold and become functional. The progress of limb regeneration has been divided into stages by Bliss (1956) who devised the use of a regeneration index [R value, (limb bud length/carapace width) × 100], which facilitates comparisons between crabs of different sizes. After autotomy there is a lag period, followed by basal growth, which establishes the basic organization of the limb. This may be followed by a plateau of anecdysis when the limb is approximately half grown. The final growth phase is proecdysial growth, controlled by the molting hormone, ecdysone (Passano and Jyssum, 1963). This is a phase of rapid growth, which may be followed by a terminal plateau immediately before ecdysis. Since the end point of regeneration is ecdysis, the presence of regenerating limbs can affect the timing of ecdysis. Factors which influence ecdysis can effect regeneration as well. Removal of eyestalks, a source of molt inhibiting hormone, is a standard way of inducing precocious molting. Such animals regenerate limbs rapidly, but generally die at ecdysis. Skinner and Graham (1970, 1972) have shown that multiple autotomy, producing many regenerating limb buds, can cause accelerated regeneration in many species of crabs, also leading to precocious molting. In previous studies (Weis, 1976a, Weis and Mantel, 1976), the fiddler crabs *Uca pugilator* and *Uca pugnax* were used to study the effects of various environmental factors on regeneration and molting after multiple autotomy. It was noted that specimens of *U. pugnax* seemed to regenerate and molt more rapidly than specimens of *U. pugilator*. However, the two species had not been studied at the same time under identical conditions. A comparative study of regeneration in several species of fiddler crabs was then undertaken. This paper is a report of a study on the regenerative rates of the temperate species, *U. pugilator* and *U. pugnax* under identical conditions, as well as the regeneration of several tropical species, *U. rapax, U. thayeri* and *U. speciosa,* as compared to *U. pugilator* from the same location.

Regeneration in fiddler crabs can be affected by environmental pollutants, such as insecticides (Weis and Mantel, 1976) and heavy metals (Weis, 1976b). Methylmercury as a pollutant of the marine environment is of great concern. It can be produced from inorganic mercury by bacterial action and is generally far more toxic than inorganic mercury. The effects of methylmercury on regeneration were tested on *U. pugilator, U. rapax,* and *U. thayeri.* Fiddler crabs, with their estuarine intertidal habitat, are subject to heavy metal pollution, especially in industrial areas.

MATERIALS AND METHODS

Specimens of *U. pugnax* and *U. pugilator* were collected from Accabonac Harbor, East Hampton, New York. Autotomy of one chela plus six walking legs was induced by pinching each merus with a hemostat. Crabs were maintained in a small amount of sea water (30‰ salinity) at 24° C in groups of 8–10 crabs per 5-liter plastic aquarium. Groups were maintained in the same location and received 14 hours of light to 10 hours darkness. Groups were arranged to have the same mean carapace width (13 mm) and sex ratio (1 : 1). Crabs were fed twice a week with Purina "Fly Chow," and the water was changed following each feeding period. Limb buds were measured twice a week under a dissecting microscope with a calibrated ocular micrometer. In all cases, the first walking leg, as a representative limb bud, was measured, and values were converted to R values. Mean R values for each group were compared by a *t*-test. Times of ecdysis were recorded for all animals. This limb bud generally attained an R value of about 20 prior to ecdysis.

Specimens of *U. pugilator, U. rapax, U. thayeri,* and *U. speciosa* were collected in the winter from Boca Raton, Florida, and treated similarly. In these experiments, however, animals were kept in small individual plastic containers in a small amount of diluted sea water (9–10‰ salinity) except when otherwise specified. Eight to ten animals of each species were used in each experiment.

In the methylmercury experiments, methylmercuric chloride (I.C.N. Pharmaceuticals, Plainview, New York) was dissolved first in 0.2% $NaHCO_3$ for a stock solution of 0.1 mg/ml and then added to the water. Control crabs in these experiments received $NaHCO_3$ in the water. Crabs were placed in the appropriate solution immediately after multiple autotomy. After ecdysis, the regenerated limbs were examined under a dissecting microscope.

RESULTS

Comparative regeneration rates

Temperate species. When maintained under identical conditions, *U. pugnax* regenerated more rapidly than *U. pugilator* of the same size (13 mm). Limb buds of *U. pugnax* reached a somewhat larger size before ecdysis, and the crabs molted soon than *U. pugilator* (Table I).

TABLE I

R values (mean ± standard error) of first walking legs of U. pugnax *and* U. pugilator *maintained under identical conditions.*

Carapace width 13 mm	Days					
	7	10	14	17	21	24
U. pugnax	2.5 ± 0.7	7.2 ± 0.9	14.2 ± 1.2	18.2 ± 1.3	20.3 ± 1.2 30% molt	90% molt
U. pugilator	1.0 ± 0.4	4.8 ± 0.6*	10.9 ± 0.9*	15.0 ± 1.0*	17.4 ± 1.1	50% molt

* $P \lesssim 0.05$.

Tropical species. The first experiment with the Florida crabs was a pilot study with *U. rapax, U. pugilator,* and *U. thayeri* of 14–16 mm carapace width. *U. rapax* regenerated more rapidly than *U. pugilator,* which regenerated more rapidly than *U. thayeri.* By 28 days, 90% of the *U. rapax,* 30% of the *U. pugilator,* and none of the *U. thayeri* had molted. The experiment was repeated with specimens of *U. rapax, U. thayeri, U. pugilator* and *U. speciosa* of 11–13 mm carapace width. The same relative rates were seen (though these smaller individuals completed ecdysis sooner than their larger counterparts in the previous experiment, as expected) and *U. speciosa* was found to regenerate at a rate equivalent to *U. rapax.* The data from the 11–13 mm crabs are seen in Figure 1a. Specimens of *U. thayeri* exhibited a relatively long terminal plateau before ecdysis. However, the overall length reached by limb buds in this species was greater than the others, R being greater than 20, rather than 18–19.

Since previous studies have shown that regeneration in *U. pugilator* is only slightly retarded in crabs maintained in groups (Weis, 1976a), experiments were done on the effects of grouping in these species. Separate groups of ten *U. pugilator, U. thayeri* and *U. rapax* (5 males, 5 females in each group) of carapace width 15–18 were placed in 5-liter plastic aquaria in shallow water. The *U. pugilator,* as expected, continued growth and molted with only slight delay. The *U. thayeri,* however, formed a plateau after basal growth (at R_1 of about 10). The *U. rapax* exhibited a lengthened proecdysial phase and terminal plateau (at R_1 of about 18); only 33% had molted by 38 or 46 days. The data from this experiment are seen in Figure 1b. At 46 days the specimens of *U. thayeri* and remaining *U. rapax* were placed into separate containers, and within two days 43% of the remaining *U. rapax* had molted. By one week the *U. thayeri* had re-commenced growth, and ecdysis began 18 days after separation.

Similar groups of *U. rapax* in their normal substrate did not exhibit this retardation but regenerated at a rate comparable to those kept in individual containers. Of a group of *U. thayeri* in the mud substrate, half formed a plateau after basal growth, and half continued growing. When specimens of *U. thayeri*

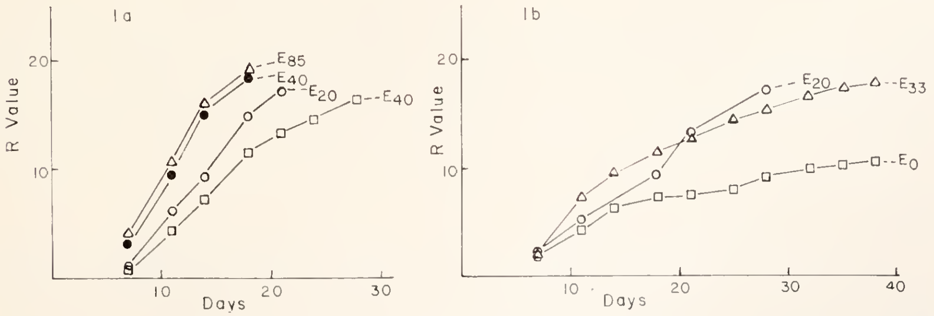

FIGURE 1. Pattern of limb regeneration: a, mean R values of first walking leg of 11–13 mm *U. thayeri* (square), *U. pugilator* (open circle), *U. speciosa* (solid circle), and *U. rapax* (triangle) after multiple autotomy and maintenance of crabs in individual containers; b, mean R values of first walking leg of 15–18 mm *U. thayeri* (square), *U. pugilator* (circle), and *U. rapax* (triangle) after multiple autotomy and maintenance of crabs in groups of ten. "E" with a subscript indicates the time at which that percentage of crabs had undergone ecdysis.

were maintained in groups of four, they again formed a plateau after basal growth, although 25% did reach ecdysis by 46 days. When they were kept in pairs, however, they regenerated and molted at the same rate as animals kept individually.

Smaller individuals of *U. rapax* (carapace width < 16 mm) did not show the long terminal plateau when kept in groups of 10 or in pairs. When specimens of *U. speciosa* (carapace width 10–13 mm) were kept in a group of 10, regeneration continued and ecdysis occurred at the same time as that of crabs kept individually.

Appearance of regenerated limbs

It has previously been reported that regenerated limbs of *U. pugilator* are light in color because, although they have the same number of pigment cells as old limbs, their melanophores are more sparsely branched and contain a smaller amount of pigment within them (Weis, 1976a). This very light pigmentation persisted when crabs were kept in the laboratory for up to three months after ecdysis. In some individuals, evidence of banding was visible microscopically. Even when animals were maintained on a dark substrate, the new limbs remained strikingly lighter than old limbs.

Newly regenerated limbs of *U. rapax* and *U. thayeri* were also much lighter in color than old limbs. The new limbs had a banded appearance, with three bands on the merus, two on the carpus and two on the propus. Old limbs were more uniformly dark. In the band regions there were approximately 120 stellate or punctostellate melanophores/mm^2, and 40–50 punctate erythrophores/mm^2. Chromatophores between the bands were sparse. Within a week the melanophores expanded to a more reticulate condition, making it difficult to distinguish individual cells. The limbs became darker in color and the banding less obvious as melanophores gradually filled in the interband region.

A similar banding pattern was seen in newly regenerated limbs of *U. speciosa*. However, the overall general appearance of the bands was brown in color due to the increased dispersion of the erythrophores, the combination of red and black producing a brown appearance. After several days, the melanophores became more dispersed and appeared in the interband regions, making them narrower, and the limb as a whole, darker, although the banding pattern remained clear. Therefore, in *U. rapax, U. thayeri* and *U. speciosa* the newly regenerated limbs gradually became almost as dark as the original limbs. In *U. pugilator,* however, the light color of the newly regenerated limbs persisted over a considerably longer period of time.

Methylmercury effects

U. rapax. In the first experiment with *U. rapax*, crabs 10–12 mm in carapace width were exposed to methylmercury at 0.001, 0.01, and 0.1 mg/liter; controls received $NaHCO_3$. Regeneration rates were comparable in controls and all experimental groups, and ecdysis began in all groups by 18 days after multiple autotomy. Subsequently, a group of *U. rapax* (12–16 mm) were exposed to 0.5 mg/liter methylmercury (Table II). Regeneration was inhibited by this concentration, and only 40% of the Hg-exposed crabs reached ecdysis by day 32. The newly regenerated limbs of control crabs had the banding pattern described previously, but

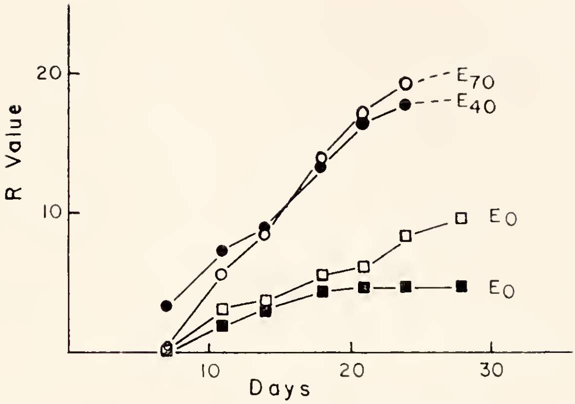

FIGURE 2. Growth of limb regenerates of first walking leg of 12 mm *U. pugilator* in 36‰ salinity (open circle), 9‰ salinity (solid circle), 0.5 mg/liter methylmercury in 36‰ salinity (open square), and 0.5 mg/liter methylmercury in 9‰ salinity (solid square). "E" with a subscript indicates the time at which that percentage of crabs had undergone ecdysis.

the regenerates of the animals that molted in methylmercury were practically devoid of pigmentation. When examined microscopically, erythrophores and leucophores were seen, but the normally more numerous melanophores were absent altogether.

When crabs (14–17 mm) were exposed to 0.3 mg/liter, growth was retarded somewhat, with 25% of the Hg-exposed and 50% of the controls reaching ecdysis by 21 days. Half of the experimental animals had incompletely regenerated one or more limbs, with distal segments lacking. Newly regenerated limbs of all experimental crabs lacked melanin. When this experiment was repeated in full strength sea water (36‰ salinity), 37.5% of the experimental crabs and 60% of the control crabs molted by 21 days. Melanin was again missing in newly regenerated limbs of all Hg-exposed crabs.

U. thayeri. In the first experiment with this species, crabs 13–16 mm were exposed to 0.1 and to 0.5 mg/liter methylmercury. The lower concentration had no retarding effect on regeneration. There was, if anything, a slight acceleration of growth. When these animals molted however, the new legs lacked melanin. Erythrophores and leucophores were present, however, and the limbs had a bright red appearance. The animals in 0.5 mg/liter showed practically no limb growth, and at 38 days after multiple autotomy, at which time their R values were only 4.1 ± 1.8, they were returned to clean water. Some showed gradual recovery, the first of these crabs reaching ecdysis 26 days later. All of them, however, lacked melanin in the new limbs.

Further experiments were performed to ascertain what length of time the crabs had to be in 0.1 mg/liter to inhibit the production of melanin. Two groups of crabs (15–18 mm) were set up: one was put into 0.1 mg/liter until the animals reached R values of about 10, then transferred to clean water; the other group was put into clean water until they reached R values of about 10, then they were transferred to 0.1 mg/liter. Crabs in this group attained R values of 10 by 12–15 days. At that time pigment was just beginning to appear in some of the regenerates. In both of these groups only 25% of the animals were lacking melanin in the new limbs after ecdysis.

Additional experiments to investigate this phenomenon involved the effect of 0.1 mg/liter of methylmercury in full strength sea water (36‰). In this experiment 50% of the crabs lacked melanophores in the regenerates. The rest had melanophores, but only one-half to two-thirds as many as in the old limbs. Normally there are about as many melanophores in the new limbs as in old ones.

Another group was placed in clean water for the first week after multiple autotomy, and then, as growth was starting, transferred to 0.1 mg/liter methylmercury. At ecdysis, melanin was absent in limbs of 80% of the animals, and those animals which had melanin had only one-half to two-thirds the normal number of cells.

U. pugilator. Experiments were performed to investigate the effects of salinity on the inhibitory action of methylmercury on regeneration. In the first experiment four groups of 12 mm crabs were used. The first two groups were controls in 9‰ and 36‰ salinity, respectively. Group 3 was exposed to 0.5 mg/liter methylmercury in 9‰ salinity, and group 4 was exposed to the same concentration of mercury in 36‰ salinity. As shown in Figure 2, regeneration was greatly inhibited in both full strength and diluted sea water containing methylmercury. Those in full strength sea water grew somewhat more than those in the diluted sea water, however, and the controls in full strength sea water molted somewhat sooner than those in the diluted water. The experimental crabs were analyzed for mercury content by atomic absorption spectrophotometry by Spectrum Laboratories Inc., Fort Lauderdale, Florida. The crabs at lower salinity had taken up more mercury (2 ppm) than those in full strength sea water (0.8 ppm).

In the next experiment four similar groups were organized, except that the concentration of methylmercury was only 0.1 mg/liter. No effect of methylmercury at this concentration, either in full strength sea water or at 9‰ could be observed. After ecdysis, however, 25% of the Hg-exposed crabs in both salinities lacked melanin in the newly regenerated limbs.

Since 0.5 mg/liter at 9‰ salinity had the greatest effect on the initiation of regeneration, a group of crabs (12–14 mm) were kept in clean dilute water until they reached R values of about 10 and then were transferred to 0.5 mg/liter to see if this concentration would inhibit further growth. Controls remained in clean water. The data (Table II) show some retardation of growth after the

TABLE II

R values (mean ± standard error) of first walking legs of (A) U. rapax *(12–16 mm) with and without exposure to 0.5 mg/liter methylmercury (B)* U. pugilator *(12–14 mm) transferred from clean water to 0.5 mg/liter methylmercury at R_1 of about 10.*

		Days							
		7	11	14	18	21	25	28	46
A	Control	2.9 ± 0.8	9.7 ± 1.9	12.0 ± 1.3	17.5 ± 1.7	19.7 ± 0.9	67% molt		
	0.5	0.8 ± 0.6	2.6 ± 1.0	4.2 ± 1.2	5.3 ± 1.6	6.0 ± 1.9	7.9 ± 2.6		33% molt
B	Experimental	2.2 ± 0.6	5.8 ± 0.6	9.1 ± 0.6*	10.1 ± 0.5	13.0 ± 1.0	14.7 ± 1.3	15.2 ± 1.9	
	Control	1.1 ± 0.3	5.5 ± 0.7	8.1 ± 0.9	13.2 ± 1.2	16.5 ± 1.1	20.0 ± 0.5	50% molt	

* Transfer to methylmercury.

addition of the methylmercury. Nevertheless, the crabs did continue growth and did molt under these conditions. Ninety per cent of the crabs in methylmercury had no melanin in their regenerated limbs. In 20%, erythrophores were also lacking. In one individual melanin formed, but melanophores were present in only one-half to two-thirds the normal amount. This was an individual whose R value had exceeded 10 at the time of transfer, and in which melanin had already formed prior to the methylmercury exposure.

In control crabs, melanin normally appears in limb buds at R values of around 10, and the first melanophores to be seen are small groups of cells on the propus and carpus. A few days later erythrophores become visible, and melanophores appear on the merus. The dactyl is the last joint to develop pigmentation.

When newly molted crabs lacking melanin were maintained in clean water, after one to two weeks melanin became visible in some individuals. Melanophores were initially very light brown and gradually became darker and more branched. These limbs became indistinguishable from newly regenerated limbs of crabs that had not been in methylmercury. Melanin did not develop simultaneously in all limbs of a given animal, but melanophores were uniformly distributed throughout the length of a limb, and did not exhibit any proximo-distal gradient.

DISCUSSION

It has been demonstrated that after multiple autotomy, specimens of *U. pugnax* regenerate and molt faster than *U. pugilator* (Table I). Fingerman and Fingerman (1974) showed in *U. pugilator* that specimens which molted sooner produced smaller regenerates. Here, however, the *U. pugnax* not only molted sooner, but produced larger regenerates as well. This species also appears "tougher" and shows less mortality in response to handling and experimental manipulation in the laboratory. Engel (1973) has found this species to be more resistant to irradiation than *U. pugilator* or *U. minax*.

U. rapax is closely related to *U. pugnax* and was previously considered a subspecies of the same species (Tashian and Vernberg, 1958). It also regenerates more rapidly than *U. pugilator,* as did *U. speciosa*. The latter species is in the same subgenus (*Celuca*) as *U. pugilator*, whereas *U. rapax* and *U. pugnax* are in the subgenus *Minuca* (Crane, 1975). *U. thayeri* (subgenus *Boboruca*) was the slowest to regenerate and generally exhibited a long terminal plateau prior to ecdysis. *U. thayeri* was observed to be much less active than the other species under laboratory conditions and has been reported to have a low general level of social activity in the field (Crane, 1975). Furthermore, it was more difficult to autotomize limbs of this species; more pressure and injury was required. *U. rapax*, on the other hand, autotomized limbs most readily. Frequently, during capture in the field, one or more appendages would be dropped without any injury having occurred. This species also appeared to be the most active. Carapace hardening after ecdysis occurred most quickly in *U. rapax* and last in *U. thayeri*. Thus, there is a correlation between readiness to autotomize limbs and speed of regeneration and molting.

U. thayeri had a higher mortality at ecdysis and arrested its regeneration after basal growth when maintained in groups. The arrest of growth in community tanks is adaptive, since crabs molting in such situations are sometimes eaten by their tank-mates. A growth arrest was seen in the land crab, *Gecarcinus*, by Bliss and Boyer (1964) but did not occur in *U. pugnax* or *U. pugilator;* the only manifestation in these crabs was a slight retardation in males when grouped (Weis, 1976a). In *U. thayeri*, however, the plateau occurred in both males and females, but did not occur when crabs were kept in pairs. Specimens of *U. rapax* in a group showed a prolonged proecdysial phase and terminal plateau, another strategy to delay ecdysis and thereby to enhance survival. A delay did not occur when specimens of *U. rapax* were maintained on a substrate which permitted them to dig burrows, or in small individuals. The difference between *U. pugnax* and *U. pugilator* on one hand, and *U. thayeri* and *U. rapax* on the other, may be adaptations to their being temperate or tropical species. Since regeneration is totally inhibited at 16° C (Passano, 1960; Weis, 1976a), temperate species spend most of the year at temperatures at which they cannot regenerate. It is therefore advantageous for them to replace lost limbs rapidly before temperatures drop to levels which will inhibit growth. Tropical species are not subject to low temperatures and can "afford" to arrest growth in group situations to increase their chances of surviving ecdysis. It is worth noting that *Gecarcinus* is also a tropical species. However, *U. speciosa*, a tropical species, did not delay growth when kept in a group. However, all individuals of this species were small, and *U. rapax* of equivalent size also did not delay ecdysis in a group. Similarly Rao (1965) has found in *Ocypode* that growth and molting in large individuals is inhibited by the presence of other crabs, but that of small individuals is not.

Differences between species of fiddler crabs in resistance to environmental stress have been noted (Vernberg and Vernberg, 1975). Many of these differences are differences between temperate and tropical species. For example, *U. rapax* is more resistant to high temperatures than *U. pugnax* or *U. pugilator*, and the latter two species are more tolerant of low temperatures than the tropical *U. repax*, *U. mordax*, *U. thayeri*, or *U. leptodactyla* (Vernberg and Tashian, 1959).

The relative rates of regeneration observed in this study are not always correlated with the relative rates of larval development. Vernberg and Vernberg (1975) have found that *U. pugilator* larvae develop to the megalopa stage faster than *U. pugnax* at 25° C and 21‰ or 30‰ salinity. However, at 20° C and 30‰ salinity, *U. pugnax* developed faster than *U. pugilator*. The tropical *U. rapax* was much slower to reach the megalopa stage than the other two species, following the general phenomenon that tropical species function more slowly than temperate zone species at a common temperature (Vernberg, 1962). In this regard, the more rapid regeneration of *U. rapax* and *U. speciosa* in the present study is an exception to this general phenomenon.

A seasonal difference in regeneration rate, especially in *U. thayeri*, was noticeable, since control *U. thayeri* used in methylmercury experiments later in the spring (March–April) regenerated more rapidly than those used earlier and showed a shorter terminal plateau. Ecdysis started by 28 days in 16–18 mm *U. thayeri* used later in the spring. The other species also regenerated somewhat more rapidly as spring proceeded. Seasonal differences in regeneration rate correlated with degree of activity observed in the field. For example, in January and February, specimens of *U. thayeri* were never observed outside their burrows, but were so observed later in the spring.

In some locations, the habitat preferences of the species studied herein are not the same. For example, in Accabonac Harbor, specimens of *U. pugnax* were found in a muddy substrate on a vertical incline, whereas specimens of *U. pugilator* were found in a sandy, more horizontal substrate. In general, *U. rapax* and *U. thayeri* are associated with higher levels in mangrove swamps, and *U. pugilator* with sandy beaches. However, in the Boca Raton collection site, all four species were intermingled in a mixture of fine sand and mangrove peat along drainage ditches extending from the Intracoastal Waterway. No stratification or segregation of species could be discerned. Burrows with chimneys, associated with *U. thayeri,* were often found with other species as occupants. Small numbers of *U. burgersi* and *U. leptodactyla* were also present at this site.

In all three species studied, 0.1 mg/liter of methylmercury had no effect on the rate of regeneration or ecdysis. However, this concentration caused a complete inhibition of melanogenesis in *U. thayeri,* partial inhibition in *U. pugilator,* and no inhibition in *U. rapax.* A mercury concentration of 0.5 mg/liter inhibited regeneration to the greatest extent in *U. thayeri* and to the least extent in *U. rapax.* Thus, the order of resistance to methylmercury is *rapax* > *pugilator* > *thayeri.* This is the same as their relative rates of regeneration, as previously noted.

Two separate effects of methylmercury were seen: inhibition of melanogenesis at lower concentrations, and inhibition of limb growth at higher levels. Inorganic mercury has also been found to inhibit limb regeneration in *U. pugilator* at 1.0 mg/liter, but to have no effect at 0.1 mg/liter. At the higher dosage, mortality rates were high (Weis, 1976b).

Shealy and Sandifer (1975) found that mercury delayed molting and extended the development time of larval grass shrimp. A possible explanation for the retardation of development and growth is that methylmercury is a mitotic inhibitor. It has been found to disrupt the spindle apparatus and cause chromosome breakage (Ramel, 1969). Mercury has also been found to decrease the metabolic rate of fiddler crabs (Vernberg and Vernberg, 1972) and to decrease their activity (Vernberg, DeCoursey and O'Hara, 1974). Larval fiddler crabs are much more sensitive than adults (DeCoursey and Vernberg, 1972); levels of mercury as low as 0.0018 ppb reduced survival of zoeae.

The greater inhibition produced by 0.5 mg/liter in diluted (9‰) sea water as compared with full strength sea water corroborates the findings of Vernberg and Vernberg (1972), who showed that fiddler crabs were more susceptible to mercury at low salinities, and that of Vernberg and O'Hara (1972), who noted that adult specimens of *U. pugilator* take up more mercury in the gills at lower salinities. In that study, total body counts of mercury at different salinities were the same, however. In the present study the total body amount of mercury was greater at lower salinity. This may be a difference between inorganic and organic mercury.

Although extensive work has been done on the physiology of chromatophores in Crustacea (Fingerman, 1965, review), the source of new chromatophores in regenerated limbs remains obscure. The absence of melanin in the mercury-exposed crabs may be due to the absence of melanophores *per se* or to the failure of melanophores to synthesize melanin, due to interference with biochemical pathways. Phenylthiourea can inhibit melanin formation in various organisms including crabs (Green, 1964), and chemicals such as sodium azide and cyanide can inhibit migration of pigment cells in salamander embryos (Child, 1950). Child discussed the possiblity that pigment formation rather than cell migration was inhibited but suggested that it was cell migration, because the pigment cells that did form were as dark as controls, and he saw only very few cells which were slightly pigmented. In the present study intermediate conditions were seen in some experiments. Generally, in partially inhibited crabs, only one-half to two-thirds the normal number of melanophores were observed in the regenerates. Normal regenerates have about the same number of melanophores/mm^2 as old limbs from the same individual, although the cells in the new limbs are less branched and have less pigment in them. The reduction in cell number might imply an inhibition of cell migration, or mitotic activity of precursors (methylmercury is a mitotic inhibitor) rather than an inhibition of melanin synthesis. However, the fact that red and white pigment usually did form might indicate that melanin synthesis was specificially inhibited, unless, in some way, the migration of melanoblasts was prevented, but that of erythroblasts and leucoblasts was not. It would seem more likely that the different biochemical pathways of pigment formation would be differentially affected. Furthermore, the delayed appearance of melanin in limbs of newly molted crabs maintained in clean water would indicate a recovery of the enzyme system of melanin synthesis rather than delayed cell migration, particularly since the melanophores were evenly distributed throughout the length of the limbs and did not exhibit a proximo-distal gradient. The cells were originally very pale brown in color and gradually darkened, reflecting progressive melanin synthesis.

In fishes, pituitary hormones necessary for physiological color change are also involved in melanogenesis. Chen, Wahn, Turner, Taylor, and Tchen (1974) found that MSH induces division of melanoblasts and differentiation of melanoblasts to melanocytes in goldfish. In fiddler crabs, individuals lacking eyestalks, a major source of chromatophorotropins, do develop normal pigmentation in regenerating limbs. This, however, does not rule out a role for chromatophorotropins in melanogenesis, since the central nervous system is another source of these hormones (Sandeen, 1950).

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SUMMARY

1. When kept under identical conditions, *Uca pugnax* regenerates limbs and molts more rapidly than *U. pugilator* from the same location.

2. The tropical species *U. rapax* and *U. speciosa* also regenerate faster than *U. pugilator* from the same location. *U. thayeri* is the slowest to replace missing limbs, the slowest to harden the carapace after ecdysis, and also requires the greatest injury before autotomy will take place.

3. When kept in groups, *U. thayeri* stops regeneration after basal growth and will not molt; *U. pugilator* is only slightly retarded when kept in groups. *U. rapax* is also affected by grouping, showing a lengthened proecdysial phase and terminal plateau, thus also delaying molting.

4. Newly regenerated limbs of *U. rapax, U. thayeri,* and *U. speciosa,* aside from being lighter than old limbs, have a conspicuous banding pattern. This pattern becomes less obvious during the week or two after ecdysis as melanophores move into the interband regions and the limb as a whole darkens due to increased dispersion of pigment in the melanophores. In *U. pugilator* the newly regenerated limbs are very pale in color and remain that way for several months under laboratory conditions, even when animals are maintained on a dark background.

5. When treated with 0.5 mg/liter methylmercury, growth was inhibited entirely in *U. thayeri.* Partial inhibition was seen in *U. pugilator,* and the least inhibition in *U. rapax.* A few individuals of *U. rapax* were able to complete regeneration and molt, but there was no melanin in the regenerated limbs.

6. Inhibition of melanogenesis in regenerated limbs was also seen in *U. thayeri* and to a smaller extent in *U. pugilator* at 0.1 mg/liter methylmercury. The lack of black pigment may be due to an inhibition of cell migration but more likely of melanin synthesis. Some of these crabs developed melanin when kept in clean water after ecdysis.

7. Seasonal differences were noted in all species, but especially in *U. thayeri.* In this species, regeneration occurred much more rapidly in March–April than in January.

LITERATURE CITED

Bliss, D. E., 1956. Neurosecretion and the control of growth in a decapod crustacean. Pages 56–75 in K. G. Wingstrand, Ed., *Bertil Hanstrom, Zoological Papers in Honor of his Sixty-Fifth Birthday, Nov. 20, 1956.* Zool. Inst. Lund, Sweden.

Bliss, D. E., and J. R. Boyer, 1964. Environmental regulation of growth in the decapod crustacean *Gecarcinus lateralis. Gen. Comp. Endocrinol.*, **4**: 15–41.

Chen, S., H. Wahn, W. A. Turner, J. D. Taylor, and T. T. Tchen, 1974. MSH, cyclic AMP, and melanocyte development. Pages 319–345 in R. Greep, Ed., *Recent progress in hormone research, Vol. 30.* Academic Press, New York.

Child, C. M., 1950. Inhibition of pigment cell migration in *Triturus torosus. Physiol. Zool.*, **23**: 1–15.

Crane, J., 1975. *Fiddler crabs of the world.* Princeton University Press, Princeton, New Jersey.

DeCoursey, P. J., and W. B. Vernberg, 1972. Effect of mercury on survival, metabolism, and behaviour of larval *Uca pugilator* (Brachyura). *Oikos,* **23**: 241–247.

Engel, D. W., 1973. The radiation sensitivities of three species of fiddler crabs (*Uca pugilator, Uca pugnax.* and *Uca minax*). *Chesapeake Sci.,* **14**: 289–291.

Fingerman, M., 1965. Chromatophores. *Physiol. Rev.,* **45**: 296–339.

Fingerman, M., and S. W. Fingerman, 1974. The effects of limb removal on the rates of ecdysis of eyed and eyestalkless fiddler crabs, *Uca pugilator. Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere,* **78**: 301–309.

Green, J. P., 1964. Morphological color change in the fiddler crab, *Uca pugnax* (S. I. Smith). *Biol. Bull.,* **127**: 239–255.

Passano, L. M., 1960. Low temperature blockage of molting in *Uca pugnax. Biol. Bull.,* **118**: 129–136.

Passano, L. M., and S. Jyssum, 1963. The role of the Y organ in crab proecdysis and limb regeneration. *Comp. Biochem. Physiol.,* **9A**: 195–213.

RAMEL, C., 1969. Genetic effects of organic mercury compounds I. Cytological investigations on *Allium* roots. *Hereditas,* **61**: 208–230.

RAO, K. R., 1965. Studies on the influence of environmental factors on growth in the crab *Ocypode macrocera* H. Milne Edwards. *Crustaceana,* **11**: 257–276.

SANDEEN, M. I., 1950. Chromatophorotropins in the central nervous system of *Uca pugilator* with specific reference to their origin and action. *Physiol. Zool.,* **23**: 337–352.

SHEALY, M. H., AND P. A. SANDIFER, 1975. Effects of mercury on survival and development of the larval grass shrimp, *Palaemonetes vulgaris. Mar. Biol.,* **33**: 7–16.

SKINNER, D. M., AND D. E. GRAHAM, 1970. Molting in land crabs: stimulation by leg removal. *Science,* **169**: 383–385.

SKINNER, D. M., AND D. E. GRAHAM, 1972. Loss of limbs as a stimulus to ecdysis in Brachyura (true crabs). *Biol. Bull.,* **143**: 222–233.

TASHIAN, R. E., AND F. J. VERNBERG, 1958. The specific distinctness of the fiddler crabs *Uca pugnax* (Smith) and *Uca rapax* (Smith) at their zone of overlap in northeastern Florida. *Zoologica,* **43**: 89–92.

VERNBERG, F. J., 1962. Latitudinal effects on physiological properties of animal populations. *Annu. Rev. Physiol.,* **24**: 517–546.

VERNBERG, F. J., AND R. E. TASHIAN, 1959. Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus *Uca.* I. Thermal death limits. *Ecology,* **40**: 589–593.

VERNBERG, F. J., AND W. B. VERNBERG, 1975. Adaptations to extreme environments. Pages 165–180 in F. J. Vernberg, Ed., *Physiological ecology of marine organisms.* University of South Carolina Press, Columbia, South Carolina.

VERNBERG, W. B., AND J. O'HARA, 1972. Temperature-salinity stress and mercury uptake in the fiddler crab, *Uca pugilator. J. Fish. Res. Board Can.,* **29**: 149–194.

VERNBERG, W. B., AND F. J. VERNBERG, 1972. The synergistic effects of temperature, salinity, and mercury on survival and metabolism of the adult fiddler crab, *Uca pugilator. U. S. Fish Wildl. Serv. Fish. Bull.,* **70**: 415–420.

VERNBERG, W. B., P. J. DECOURSEY, AND J. O'HARA, 1974. Multiple environmental factor effects on physiology and behavior of the fiddler crab, *Uca pugilator.* Pages 381–425 in F. J. Vernberg and W. B. Vernberg, Eds., *Pollution and physiology of marine organisms.* Academic Press, New York.

WEIS, J. S., 1976a. Effects of environmental factors on regeneration and molting in fiddler crabs. *Biol. Bull.,* **150**: 52–62.

WEIS, J. S., 1976b. Effects of mercury, cadmium, and lead salts on limb regeneration and ecdysis in the fiddler crab, *Uca pugilator. U. S. Fish Wildl. Serv. Fish. Bull.,* **74**: 464–467.

WEIS, J. S., AND L. H. MANTEL, 1976. DDT as an accelerator of regeneration and molting in fiddler crabs. *Estuarine Coastal Mar. Sci.,* **4**: 461–466.

Reference: *Biol. Bull.,* **152**: 275–294. (April, 1977)

SEASONAL CHANGES IN THE COMMUNITY STRUCTURE OF THE MACROBENTHOS INHABITING THE INTERTIDAL SAND AND MUD FLATS OF BARNSTABLE HARBOR, MASSACHUSETTS

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Since the pioneering works of Peterson (1918) and Thorson (1957), most of the attention of workers in the field of marine benthic ecology has been directed to short-term sampling surveys of particular environments in an attempt to delimit given macrobenthic species assemblages or communities. These investigations have generally concluded that given species assemblages are greatly influenced by sedimentary parameters (*e.g.,* grain size), and that the communities are not tightly functioning units but rather are composed of individualistically distributed species which respond to a complex set of environmental and biological parameters. The result of such findings is that given particular sedimentary parameters, *a posteriori* predictions of what faunal assemblages will occur at a particular site are possible. However, over longer periods of time, the predictive value of determining the distribution of given species groupings is greatly reduced. Any naturally occurring community of organisms is not only distributed in space but also in time. To better understand patterns of community organization, therefore, more long-term studies are needed to provide basic information regarding temporal community structure and to elucidate those factors affecting species compositional change.

Studies on temporal changes in community structure and species composition are not common. Of these studies (*e.g.,* Sanders, 1960; Pearson, 1971, Tenore, 1972; Lie and Evans, 1973; Watling, 1975; Levings, 1975), most are concerned with subtidal environments. Surprisingly, there are fewer studies dealing with time-related community changes in intertidal environments (*e.g.,* Bloom, Simon and Hunter, 1972; Johnson, 1970; Holland and Polgar, 1976). Intertidal environments provide an excellent opportunity to study the effects of seasonal change, since physical variables fluctuate with greater amplitude than in bordering subtidal habitats, thus subjecting organisms inhabiting them to greater amounts of stress.

The purpose of this study is to describe the general macrobenthic community structure of a portion of the intertidal sand and mud flats of Barnstable Harbor, Massachusetts, and also to examine in greater detail seasonal changes in patterns of community structure and species composition at one of the sedimentary environments. This study is presented to provide a context for a more detailed examination of methods of resource allocation in deposit-feeding faunal assemblages and micro-dispersion patterns of surface-feeding polychaete annelids.

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Materials and Methods

Barnstable Harbor, located on the northern shore of Cape Cod, is a shallow tidal enbayment about 5.5 km long and 1.2 km wide. Over the past several decades a relatively large amount of research has been directed towards various aspects of the marsh ecology (see Redfield, 1972, for a partial list of references and an excellent description of the general development of the marsh system). The mean tidal range is about 2.9 m and approximately 60% of the tidal flats, where this study took place, are exposed at low tide (Ayers, 1959).

The physical environment of the region is typical of intertidal regions along the southern New England coast of North America. Water temperatures range from about 20° C during July to September to about 0–1° C during December to February. Sediment temperatures range from about 30° C in August to 1–2° C in December to February. The winter of 1974–1975 was unusually mild, and accumulation of ice on the flats was never observed. Ice buildup on the flats normally occurs from January to February (Green and Hobson, 1970; personal observations in the winter of 1975–1976). Ayers (1959) reports that salinity near the present study area varies between 29.6 to 31.7‰, with very little freshwater drainage in the harbor.

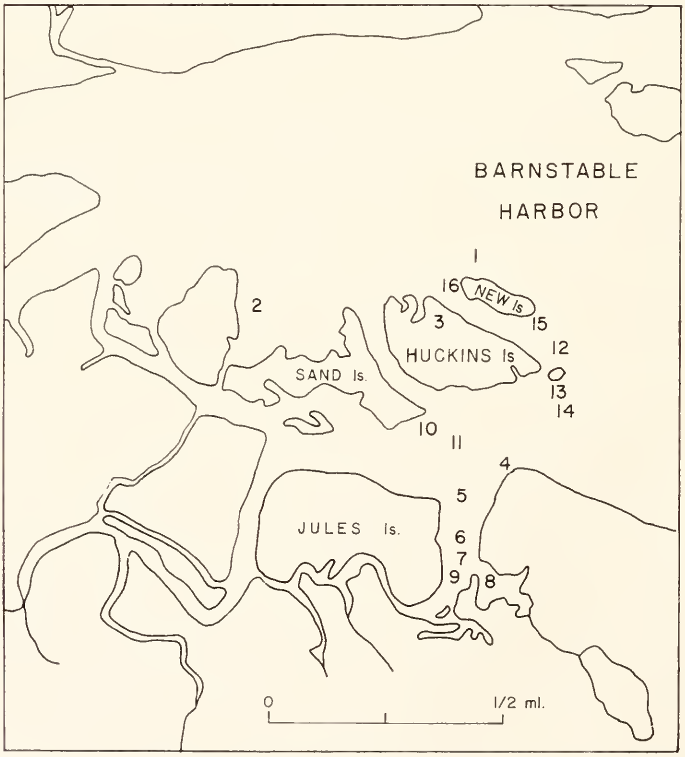

Figure 1. Map of western Barnstable Harbor, Massachusetts, indicating sampling localities.

The currents of Barnstable Harbor are controlled almost exclusively by the tides. Due to the large tidal range, strong currents occur in the harbor. Both Ayers (1959) and Sanders, Goudsmit, Mills, and Hampson (1962) have measured the currents at different regions within the harbor and found maximums from 30–80 cm/sec occurring during flood tides. The strongest currents occur just before the time of maximum ebb tide. The flats within the harbor, however, are not greatly affected by the strong currents, since they are always exposed before the time of maximum ebb current.

Changes in sediment accretion and erosion were monitored over a yearly period at stations 1 and 2 (Fig. 1). Results indicate minimal changes in the level of substratum at these localities during a 15 month period (on the order of 1–3 cm). Maximum sediment fluctuation occurred in the spring. Larger amounts of sediment movement were common, however, in the tidal creeks and areas closer to the mouth of the harbor.

Sixteen preliminary stations were sampled in the western portion of the intertidal sand and mud flats in June–July 1974. Faunal data collected from these stations were used to help in delimiting the general characteristics of the various macrofaunal assemblages of this region. One station was chosen from the initial sampling program for more detailed long-term sampling.

Samples were collected at low tide with a small hand held core 12.5 cm in diameter. The core was gently pushed into the substrate to a depth of 17–20 cm and removed with a shovel. The location of sampling sites at each station was determined by random sampling coordinates. Samples were brought to the laboratory and gently washed through a 250 μm mesh screen. The residues were preserved in 70% alcohol, sorted under a dissecting microscope and the organisms encountered were identified to species.

A small core (3.5 cm diameter) was used to sample surface sediment for substrate analysis. Sediment size-distributions were determined for each sample using standard wet-sieving analysis and mean particle size, and sorting coefficients were graphically determined from formulae of Folk (1966). The percentage of oxidizable organic carbon was estimated by the Walkley-Black wet oxidation method (Morgans, 1956).

Faunal data were subjected to classification and cluster analysis in an attempt to delimit community structure, species composition and seasonal community dynamics of the benthic macrofauna. This type of analysis has received extensive attention in marine community studies (*e.g.*, Lie, and Kelley, 1970; Stephenson and Lance, 1970; Hughes and Thomas, 1971; Stephenson, Williams and Cook, 1972; Boesch, 1973), and these authors have outlined the various methods.

In this study, the per cent similarity coefficient (a derivative of the Czekanowski similarity coefficient) was used. Stephenson *et al.* (1972) have briefly reviewed the characteristics of the different classification measures. The per cent similarity measure tends to be biased toward the more dominant and ubiquitous species, so that rarer species contribute little to the index. Preliminary data analysis comparing the fanual data with and without dominant forms, however, revealed that the clusters were not appreciably altered.

Two clustering strategies were initially employed: first, the more common group-average or unweighted pair-group method; and secondly, the newer and more popular flexible sorting strategy. Lance and Williams (1967) and Williams (1971) have reviewed the basic properties of the two strategies. After initial data processing, it was found that the group-average clustering technique yielded the more instructive classification. The "flexible" method is a strong sorting strategy (William, 1971) which increases the distance between groups as the number of elements which they contain increases. While this heightens differences between groups, it tends to divide these elements into groups that may obscure the important characteristics of individual systems. (Comparisons of the two clustering strategies are available upon request.)

Both normal and inverse classification analyses were used in the study. Normal analysis (sometimes called Q-analysis) treats samples as individuals, each being composed of a number of attributes—the various species from a given sample. Normal analysis is instructive in helping to ascertain community structure and to infer specific ecologic conditions between sampling stations from the relative distributions of the species. Inverse classification (termed R-analysis) is based on species as individuals, each of which is characterized by its relative abundance in the various samples. This type of analysis is commonly used to reveal species groups within particular habitats.

Shannon's formula (Shannon and Weaver, 1963) expressed as: $H' = -\Sigma p_1 \log_2 p_i$, where p_i is the proportion of individuals belonging to the *ith* species, was used to estimate species diversity. Evenness, the distribution of the individuals among the various species, was computed as: $J' = H'/\log_2 s$, where s is the number of species in the sample (Pielou, 1966).

RESULTS

Sediments

A summary of the sedimentary features of the sampling localities is given in Table I. Four distinctive substrate types are evident: sandy sediment (stations 10–16), muddy-sand sediment (stations 1–2), muddy sediment (stations 3 and 5–9), and gravel-sand sediment (station 4).

The muddy stations are all located in slightly higher and more protected areas of the marsh and are relatively free from tidal motion. These sediments are characterized by both high silt-clay fractions and organic carbon content. Dead and decaying fragments of *Spartina alterniflora* are commonly found throughout these sediments.

The sandy stations can be further subdivided into two categories: fine (stations 12–14) and coarse (stations 10, 11, 15, 16) sand. Coarser sand is generally found closer to the tidal channels. The sands in these areas are moved freely by tidal currents, and the surface is commonly marked by sand ripples indicating sediment instability. The finer sand stations are further from the channels where current velocities are lower. These sediments are generally well-sorted and contain low percentages of organic carbon.

The very large sand flats north of New and Sand Islands (Fig. 1) are composed of well-sorted muddy-sands. These areas are more protected from current flow across the flats and any surficial ripple marks are normally the result of wave action. These sandy flats have slightly higher amounts of organic carbon

Table I

Sedimentary and trophic group parameters from the sampling stations at Barnstable Harbor (see Figure 1 for station locations).

Station number	Per cent silt-clay	Mean phi	Phi of sorting	Per cent carbon	Deposit-feeders*		Suspension-feeders*	
					Number species	Number/ $m^2(\times 10^3)$	Number species	Number/ $m^2(\times 10^3)$
1	11.9	2.48	0.18	0.48	19	45.7	2	1.30
2	20.9	2.47	0.30	0.78	12	39.0	0	—
3	91.0	4.09	0.40	1.52	3	52.2	0	—
4	1.3	0.53	0.83	0.37	12	6.3	1	0.08
5	48.6	3.10	0.33	0.57	15	33.3	0	—
6	34.2	3.00	0.33	0.66	9	18.7	0	—
7	88.7	4.22	0.45	1.49	6	35.8	0	—
8	87.6	4.10	0.38	1.54	11	21.1	0	—
9	80.4	3.81	0.37	1.35	11	23.5	0	—
10	4.1	1.90	0.35	0.10	8	2.4	3	2.00
11	2.5	1.78	0.40	0.07	4	0.9	1	0.20
12	11.4	2.15	0.48	0.32	17	30.3	2	104.90
13	7.9	2.13	0.45	0.33	9	11.9	3	44.80
14	7.5	2.05	0.43	0.31	9	4.5	2	192.90
15	2.1	1.47	0.38	0.22	7	6.5	2	28.20
16	2.6	1.60	0.32	0.09	13	8.2	1	0.08

* Feeding types determined from examination of gut contents, Sanders *et al.* (1962) and Barnes (1964).

and silt-clay fractions when compared to the sand areas. Monthly sediment samples at station 1 indicated no significant temporal change in mean grain size or sorting coefficient according to analysis of variance ($P > 0.05$).

The one station located on the south shore of the harbor consists of gravel, sand, and clay, all thought to be derived from erosion of the upland (Redfield, 1972). This sediment is very poorly sorted, with low organic carbon content. The presence of patches of scattered stones and gravel characterizes this very heterogeneous substrate.

General community description and species composition

Table II lists the macrofaunal species occurring in 64 core samples obtained from the preliminary sampling of 16 stations in Barnstable Harbor. Of the 47 species found in the survey, the majority were polychaetes (29 species), crustaceans (9 species) and molluscs (6 species). Individual replicate samples taken at each locality were not lumped together for use in the classification analysis so as to obtain an idea of within-habitat sampling variability. The results of the inverse classification analysis used to delimit species groups, however, are based only on those species which occurred more than five times in the survey. Species eliminated from the analysis include ten which occurred once, five which occurred twice, and only one which occurred three times.

Normal analysis. Figure 2 indicates that the samples are clustered into five major groups. Group A is composed of stations 12–15 containing clean, well-sorted sands with a low silt-clay fraction (2–5%). Group B, composed of samples

TABLE II

Macrobenthic species sampled at Barnstable Harbor, June–July, 1974.

Identification number	Species	Station															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Polychaeta																	
1	*Streblospio benedicti*	X	X	X	X	X	X	X	X	X	X		X	X	X		X
2	*Eteone heteropoda*	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
3	*Lumbrinereis tenuis*	X	X		X	X		X	X	X			X				
4	*Capitella capitata*	X		X	X		X		X	X	X		X		X		X
5	*Glycera dibranchiata*									X							
6	*Polydora ligni*			X	X	X											
7	*Phyllodoce mucosa*	X	X							X			X				
8	*Heteromastus filiformis*	X	X	X	X	X	X	X	X	X	X	X	X		X		X
9	*Scolecolepides viridis*			X		X											
10	*Scoloplos acutus*	X				X						X	X	X	X	X	X
11	*Spio setosa*	X	X		X	X	X							X			
12	*Scoloplos robustus*						X	X									
13	*Clymenella torquata*					X			X								
14	*Nereis arenaceodonta*	X	X			X			X	X			X				X
15	*Pectinaria gouldii*	X	X		X	X	X										
16	*Scolelepis squamata*	X	X										X				X
17	*Tharyx* sp.	X	X		X								X				X
18	*Nephtys picta*	X															
19	*Syllides verrilli*	X	X			X	X	X	X	X	X		X	X	X		X
20	*Spiophanes bombyx*	X	X								X	X	X		X		X
21	*Prionospio heterobranchia*	X			X					X			X				
22	*Paraonis fulgens*																X
23	*Aricidea jeffreysii*	X															
24	*Eteone longa*													X		X	
25	*Nereis virens*				X					X							
26	*Nephtys caeca*										X						X
27	*Amphitrite ornata*					X											
28	*Pygospio elegans*										X			X	X	X	
29	*Chaetozone* sp.															X	
Mollusca																	
30	*Hydrobia* sp.	X											X	X	X	X	
31	*Nassarius obsoletus*	X		X		X			X				X				
32	*Gemma gemma*	X									X		X	X	X	X	
33	*Mya arenaria*	X			X						X	X		X	X		X
34	*Retusa canaliculata*	X	X														
35	*Polynices duplicatus*	X															
Crustacea																	
36	*Diastylis polita*															X	
37	*Ampelisca abdita*													X			
38	*Oxyurostylis smithi*	X															
39	*Edothea triloba*	X				X			X								
40	*Crangon septemspionosus*								X	X							
41	*Corophium insidiosum*												X				
42	*Gammarus* sp.										X		X				
43	*Unicola irrorata*		X														
44	*Ampithoe rubricata*					X	X		X								
Hemichordata																	
45	*Saccoglossus kowalewskii*																X
Oligochaeta																	
46	*Phallodrilus monospermathecus*												X	X		X	
47	*Monophylephorus irroratus*				X												

collected from stations 10, 11, and 16, is also a clean sand environment. These sediments, however, are coarser-grained with a smaller silt-clay fraction (2–3%). Group C contains the muddy sediment localities (stations 3 and 5–9) of Jules Island and Huckins Island. The major sedimentary features of these stations include high quantities of silt-clay (10–70%), small median grain size and high organic carbon content. The muddy-sand stations (1 and 2) fall into Group D. While sation 1 is more characteristic of a sandy sediment type, the larger silt-clay fraction makes it more similar to station 2 in sedimentary composition. Finally, Group E contains the very poorly sorted gravely-mud station.

Inverse analysis. The 32 species found at the various localities in Barnstable Harbor were divided into 10 groups (Fig. 3) by the inverse classification analysis.

Groups 1, 3, 5, and 7 are comprised of species which characteristically occur in clean sand areas. While normal classification analysis divided the sandy stations into two separate groups, fine and coarse sand, species groups generated by the inverse analysis indicate that degree of within-habitat species fidelity is low. About 39% of the species in these groups are found in both coarse and fine sands—the most striking visible difference being the presence of very large numbers of the small venerid bivalve *Gemma gemma* in the fine sands.

Group 4 is composed of the most ubiquitous and numerically most important species of Barnstable Harbor. *Streblospio benedicti, Eteone heteropoda,* and *Heteromastus filiformis* occur at each sediment type sampled. At sandy-mud and mud stations, *S. benedicti* and *E. heteropoda* were always ranked as the first and second most dominant species, respectively.

Groups 8 and 10 consist of species normally associated with sandy-mud and mud stations. Although normal classification analysis separated these two sediment types into discrete groups, the inverse analysis showed that between habitat species co-occurrence is high. Only two species, *Scoleocolepides viridis* and *Scoloplos robustus,* in these two groups were restricted to just one sediment type.

Group 9 consists of two species which are largely restricted to station 4, the most hetrogeneous substrate type. While the large nereid polychaete, *Nereis virens,* was also found at station 9, its greatest abundances occurred at station 4. The oligochaete, *Monophylephorus irroratus,* was never found to occur at any of the other 15 stations sampled.

Groups 2 and 6 contain just one species each. *Ampithoe rubricata,* a small amphipod, and the epifaunal tectibranch, *Retusa canaliculata,* were found in the muddy-sand stations 1 and 2. Both species were relatively rare in abundance.

In summary, inverse classification analysis produced four basic species groups: sandy faunal groups composed of species inhabiting both coarse and fine sand sub-

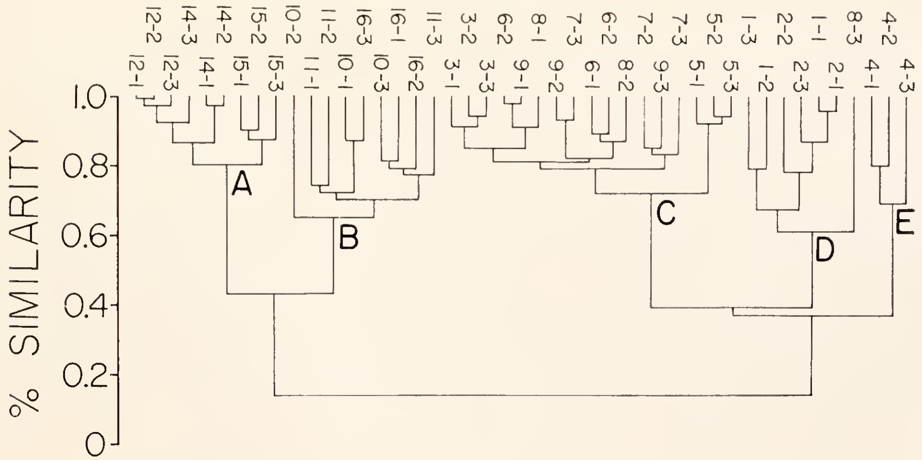

FIGURE 2. Dendrogram resulting from normal classification analysis of 16 sampling localities at Barnstable Harbor. First characters indicate sampling station (see Fig. 1); last digit indicates sample number.

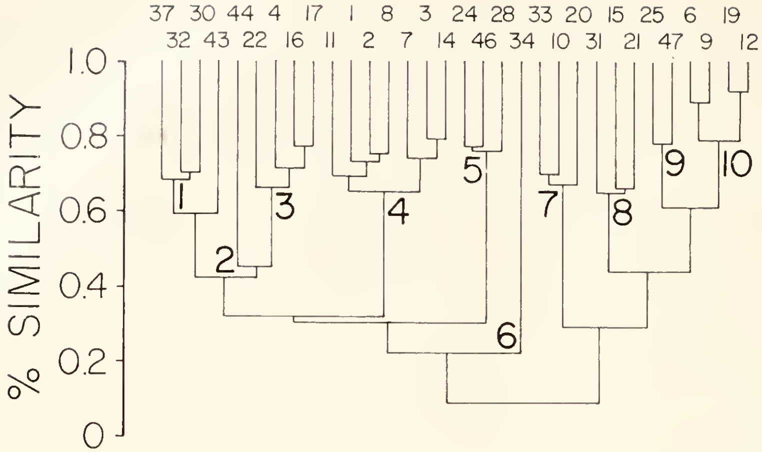

FIGURE 3. Dendrogram resulting from inverse clustering of species at the sampling stations. Species are indicated by code numbers (see Table II).

strata; ubiquitous forms characterizing the dominant faunal components of Barnstable Harbor; muddy and sandy-mud species; and single species groups which are both rare and restricted to a single sediment type.

Animal-sediment relationships. While more detailed information will be presented in a later paper (in preparation) on the feeding ecologies of the dominant benthic macrofauna, it is interesting to examine the general trophic-group sediment-type relationships found at Barnstable Harbor. Table I lists information regarding the abundance and species number of the two major trophic groups: suspension- and deposit-feeders. The majority of the benthic fauna at Barnstable Harbor are comprised of deposit-feeding species. *Mya arenaria* and *Gemma gemma* are the two most abundant suspension-feeding organisms. These forms dominate at stations 12–15 which contain well-sorted, relatively fine sands with low silt-clay and organic carbon values. *G. gemma* comprises over 99% of the abundance of the suspension-feeding forms at these stations. *G. gemma* is widely distributed and abundant in fine sands. In coarser sand localities, where surface rippling becomes increasingly pronounced, the bivalve density is low. Sediment instability resulting from waves and currents readily move this small bivalve and are probably responsible in determining its distributional pattern. *M. arenaria* is much rarer and comprises less than 1% of the total number of suspension-feeders at the sampling stations. Bradley and Cooke (1959) and Sanders *et al.* (1962) have hypothesized that high density populations of *G. gemma* inhibit larval settling of *M. arenaria.* Deposit-feeders predominate at stations 1–9 where both organic matter and silt-clay fractions are the highest. The coarse sand, ripple-marked stations (10, 11, 16) show reduced numbers and species diversity of both trophic groups.

Seasonal changes in community structure and species composition

Station 1 was sampled at approximately monthly intervals for a period of 19 months to obtain information on the degree of temporal fluctuation in both community structure and species composition.

While the basic unit of structure in any natural community is the individual, examination of the total species complement of a particular assemblage or community of organisms can provide insight into distinguishing temporal changes of community organization. Figure 4 summarizes three such measures of community structure at station 1: species diversity (H′), evenness (J′), and

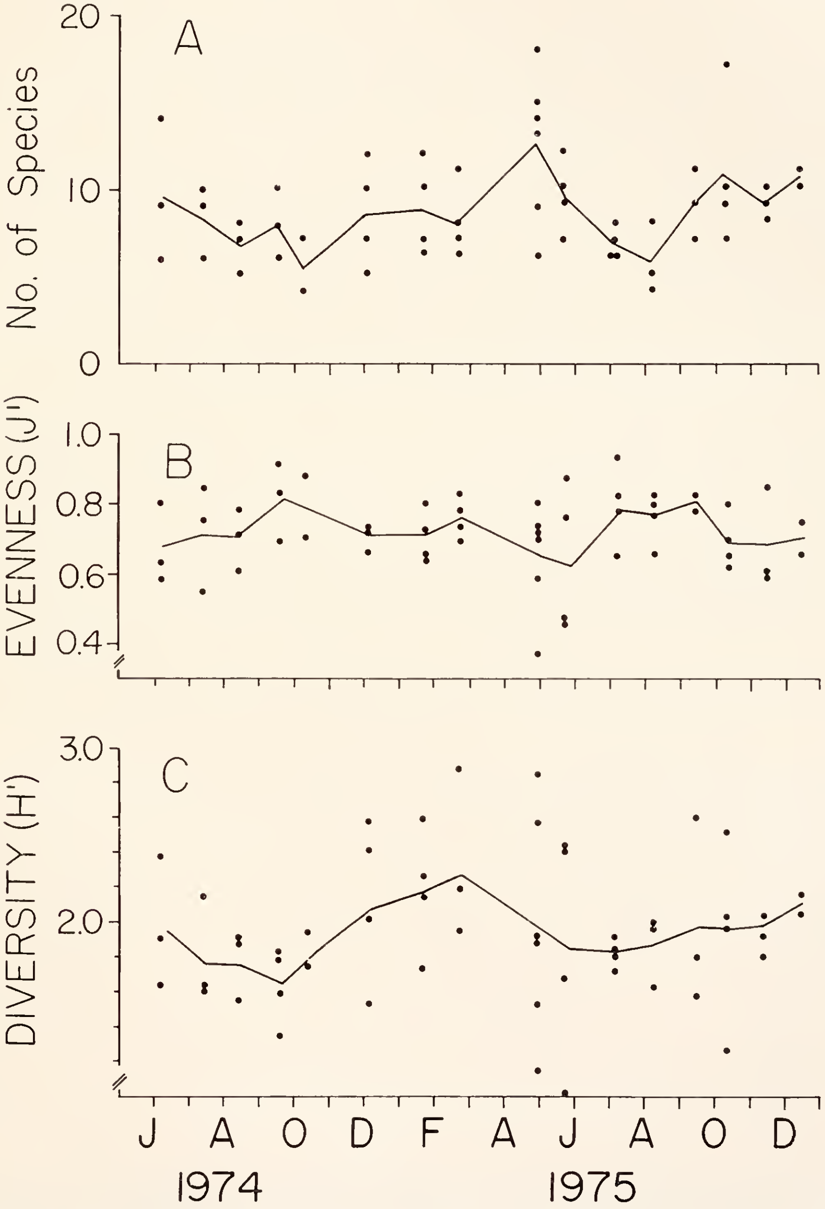

FIGURE 4. Temporal changes in number of species/245 cm² (A); evenness (B); and species diversity (C) at station 1.

number of species. While all three measures show some temporal variation throughout the sampling period, they did not vary significantly according to the results of one-way analysis of variance ($P > 0.05$). It should be noted, however, that indices similar to the ones used are not sensitive to subtle changes in species composition. In particular, Shannon's index H′ is biased toward the more dominant species and the rarer members contribute little to the measure. Also, these measures do not provide any information regarding what species are actually characteristic of a given sample(s) during different periods of the year. For these reasons classification analysis was adopted to examine the degree of temporal partitioning of samples collected throughout the year and to elucidate changes in species composition at station 1.

Normal analysis. Figure 5 presents the results of normal classification analysis of 46 samples collected from June 1974 to December 1975. Generally the major groupings derived from this procedure are subdivided by seasonal similarity and can be grouped into four clusters. Group A is largely a "winter" cluster comprising samples collected from October to January. Group B contains collections from March–May and can be regarded as a "spring" cluster. Groups C and D can be classified as "summer" and "fall" groups, respectively, though the level of separation of these two clusters is higher than found in Groups A and B.

It is evident that there is a certain degree of sample overlap between the clusters, indicating spatial variation in species abundance and composition of individual samples. Group B (spring cluster) contains one sample collected during the fall (October, 1975). Groups C and D have the greatest amount of sample overlap between clusters; each contain two samples that are not similar on a seasonal basis with other samples in the group. In all cases, upon examination of the original data matrix these samples were characterized by the absence of the rarer species which normally had occurred at other times of the year in the other samples.

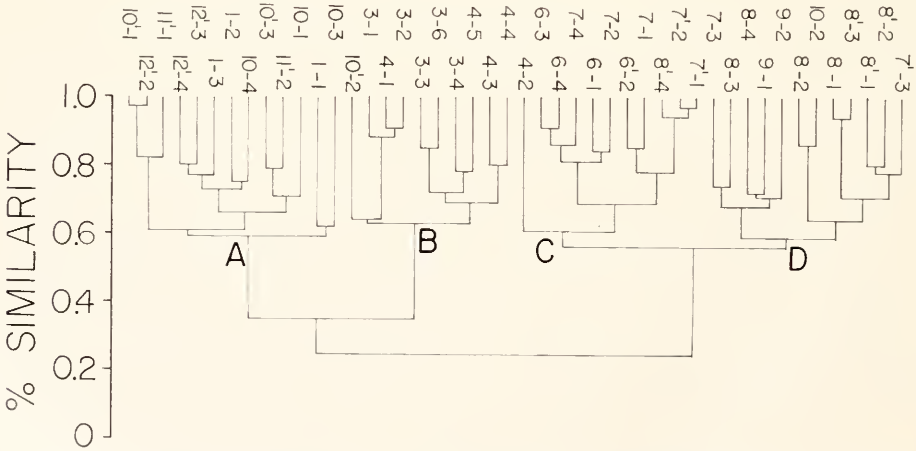

FIGURE 5. Dendrogram resulting from normal analysis of sampling station 1 from June, 1974 to December, 1975. First characters indicate the month sample was collected (prime numbers = 1975). Last number indicates sample number.

Inverse analysis. Species that occurred less than five times during the sampling program were excluded from the inverse analysis. Those species eliminated included six species that occurred only once, two forms occurring twice, and one species occurring three times. Twenty-one species were included in the analysis.

Again, four basic seasonal species groupings are evident at station 1 (Fig. 6). As would be expected, individual clusters are composed of species whose seasonal peak population abundance levels coincide with one another. Table III provides information on the time of the highest numerical abundance coupled with data on the reproductive activities of each species included in the analysis. Data on reproductive behavior of each species were compiled from published sources and personal observations. Unfortunately, some of these data are based on limited observations. In general, however, there is a close concordance between the peak population abundance levels of species and their periods of reproductive activity. In most instances there is a one to two month delay between the observation of reproductively active adults and the appearance of the new year class into the benthic population. This time lag may either be associated with the planktonic larval phase exhibited by most of the species in this study or may be the result of sampling bias. Although the use of smaller meshed screens proved impractical in this study, they may provide a more exact time scale of larvae entering the benthic populations.

Species group 1 is composed of organisms whose recruitment generally occurs in the spring and early summer months. This group contains those faunal elements which are normally the dominant components of this station. Group 2 contains species that are generally most abundant during the warmest fall months of the year. Four species of polychaetes and one species of amphipod, whose peak abundances occur in the coldest months of the year, comprise Group 3. Group 4 contains six species whose recruitment of individuals into benthic populations occurs during the late summer months of July–September.

Population characteristcis of the dominant species. Figure 7 shows a summary of the seasonal changes in the population abundance of five of the most

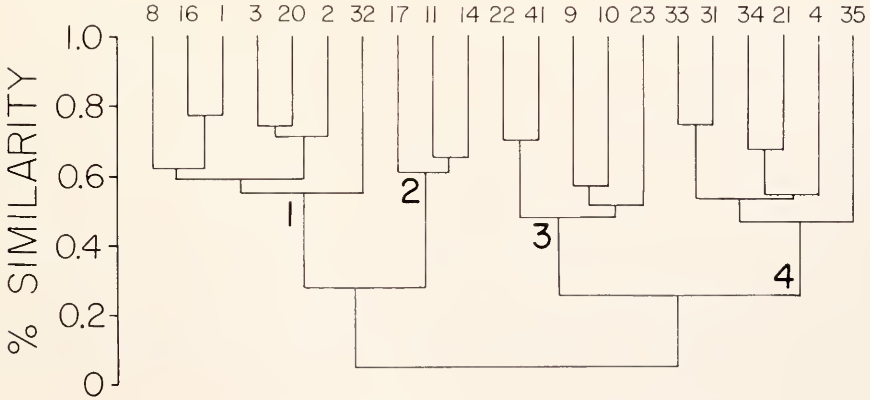

FIGURE 6. Dendrogram indicating seasonal species groups at station 1 using inverse classification analysis. Species are indicated by code numbers (see Table II).

numerically important species at station 1. *S. benedicti,* generally the most abundant and ubiquitous polychaete found in Barnstable Harbor, comprises 20–70% of the total fauna at station 1. Abundance patterns of this small spionid are highly seasonal with greatest numbers occurring in early spring. Rapid decreases occurred in the summer months in both 1974 and 1975. Much of this decline is attributable to the loss of larger individuals from the population. Size-frequency distributions of this organism are uni-modal, characteristic of annual species. The new-year class enters the population in June and continues to grow throughout the winter and following spring (unpublished). Watling (1975) and Jones (1961) found similar seasonal abundance patterns of *S. benedicti* in Delaware and southern California populations, respectively. Watling (1975) listed size data indicating the annual nature of this species.

E. heteropoda and *H. filiformis* also appear to be relatively short-lived. Size-frequency distributions of *E. heteropoda* indicate three separate modes, suggesting at least three year classes are found in the population. The 0-year class enters the population in July and continues to grow throughout the year. The population abundance of *H. filiformis,* on the other hand, was quite stable throughout the sampling program. Changes in the size-frequency distributions of this species indicate the 0-year class enters the benthic population in July–August. The size distributions also indicate two distinct size classes, suggesting first and second year groups. Buchanan and Warwick (1974) found a similar life history pattern in *H. filiformis;* however, 0-year groups appeared in January on the coast of Britain.

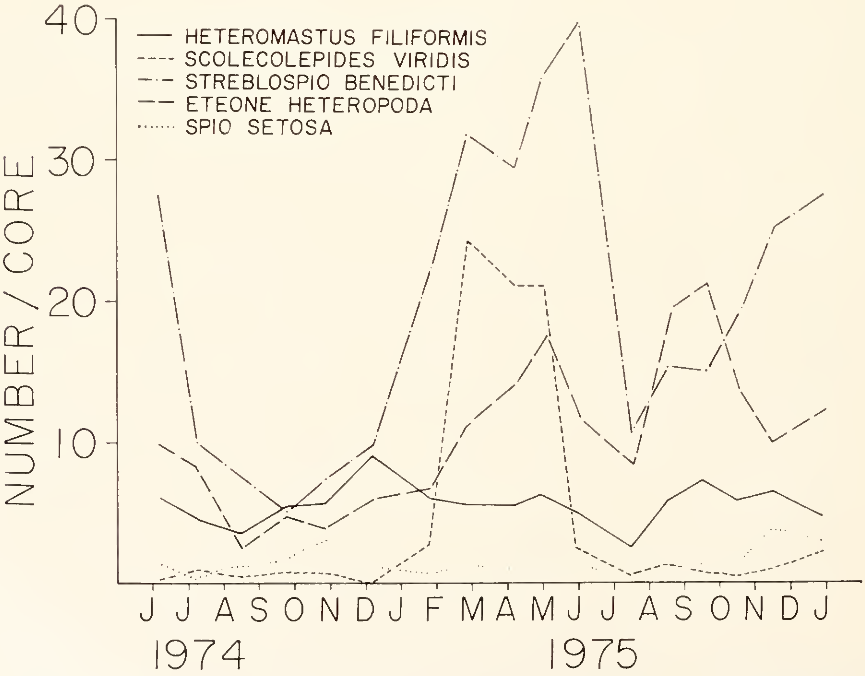

FIGURE 7. Estimated abundance (number per 245 cm²) of the five most common macrobenthic species at station 1 from June 1974 to December, 1975.

Table III

Seasonal species groups at Barnstable Harbor, time of peak population abundance, reproductive activity, and geographic range along the eastern coast of North America.

Group	Species	Peak Abundance	Reproductive activity*	Range**
1	*Streblospio benedicti*	June	June–Oct.	C
	Heteromastus filiformis	July–Aug.	June–July	C
	Scolelepis squamata	April–June	Mar.–June	C
	Lumbrinereis tenuis	May–June	June	C
	Spiophanes bombyx	May–June	June–Aug.	C
	Eteone heteropoda	June–July	June (?)	C
	Gemma gemma	May–June	May–Aug.	C
2	*Tharyx* sp.	Sept.–Oct.		V
	Nereis arenaceodonta	Aug.–Oct.	Aug.	V
	Spio setosa	Sept.	Oct.–Feb.	V
3	*Scolecolepides viridis*	Feb.–May	Oct.–Nov.	N
	Scoloplos acutus	Feb.–April	Feb.–July	N
	Aricidea jeffreysii	April–May	July	C
	Paraonis fulgens	Jan.–Feb.		N
	Corophium insidiosum	April–May	April–Aug.	N
4	*Mya arenaria*	Sept.–Oct.	Sept.–Oct.	C
	Nassarius obsoletus	Aug.–Sept.	June–July	C
	Retusa canaliculata	Aug.–Sept.	June–Aug.	C
	Prionospio heterobranchia	July	April–July (?)	V
	Capitella capitata	July–Aug.	April–Nov.	C
	Polinices duplicatus	Aug.–Sept.		V

* Information obtained from: personal observations; Simon and Brander, 1967; Pettibone, 1963; George, 1966; Dean, 1965; Grassle and Grassle, 1974; Scheltema, 1962; Turner, 1951; Grant, 1965.

** Biogeographic range of species: C = generally ranging along entire North American coast; V = generally occurring from Massachusetts to Florida; N = normally ranging from Maine to Massachusetts. Information from: Pettibone, 1963; Day, 1973; Foster, 1971; Abbott, 1954; Bousfield, 1973.

The large spionid, *Spio setosa,* showed two separate population abundance peaks, one which occurred in November and a slightly larger one which occurred in May (Fig. 7). *S. setosa* appears to exhibit two reproductive periods: first, a spring period in which eggs are laid in female tubes and develop into benthic larvae; and secondly, a fall period in which pelagic larvae are abundant (Simon, 1967, 1968). The two peaks in abundance found at station 1 probably reflect the two recruitment periods.

Figure 7 shows that *Scolecolepides viridis* is most abundant during February–May. During the remainder of the year, a small and relatively stable population level is found at station 1. George (1966) reports that *S. viridis* spawns from October–November in Nova Scotia, Canada, waters, and reproductive activity appears to be inversely correlated with increases in water temperature.

Discussion

As with other benthic studies, sedimentary parameters appear to be influencing general trophic and species composition of the macrobenthos at Barnstable Harbor.

The work of Sanders (1958, 1960) has brought into focus the importance of sedimentary features that influence the distribution of suspension- and deposit-feeding benthos. From examination of the theoretical properties of sediment dynamics, Sanders postulated a value of 0.18 mm as the optimal grain size for supporting suspension-feeders. He also suggested that high silt-clay fractions in sediments (relating to greater amounts of food) helped to explain the presence of the deposit-feeding benthos. Though these predictions have been successfully tested in the majority of subtidal surveys (but see McNulty, Work and Moore, 1962), they show inconsistencies when applied to intertidal studies (Sanders, *et al.*, 1962; Bloom, *et al.*, 1972). Examination of Table I indicates that areas dominated by suspension-feeders (though they normally comprise 1–2 species) have an average median grain size of 0.28 mm, larger than Sanders' predicted value. The importance of high silt-clay fractions influencing the distribution and abundance of deposit-feeders, however, is very evident in this table.

Sanders (1958) also suggested that the distribution of suspension-feeders is greatly effected by sediment instability (median grain size greater than 0.18 mm). Data from most coarse sand stations (10, 11, and 15) at Barnstable Harbor indicate that the abundance of both deposit- and suspension-feeders is sharply reduced. These stations are characterized by pronounced surficial ripple marks, evidence of sediment instability. This instability coupled with low amounts of organic carbon and silt-clay fractions explain the reduced abundances of both trophic groups in these areas.

The survey of Sanders *et al.* (1962) of five locations on or near Huckins Island, Barnstable Harbor, found that two sediment types (muds and stable sands) were dominated by deposit-feeding trophic *biomass* at four of the stations and did not fit the predictions stated above. Re-examination of the data, however, indicates that the *numerical abundance* of the two trophic groups tends to be correlated with amounts of silt-clay. Stations A and C (Sanders *et al.*, 1962) dominated by *G. gemma*, have the lowest amount of silt-clay. Deposit-feeders dominate at those stations with higher percentages of silt-clay. Median grain size relationships, however, show no significant trends in predicting benthic trophic structure. The reasons for the discrepancies in the two sets of data are unknown. Comparisons of both sedimentary parameters and rank order abundance of the macrobenthos indicate major long-term (over 15 years) changes at all localities have taken place.

While examination of temporal changes at one sedimentary type at Barnstable Harbor revealed distinct seasonal change in species composition, no patterns in species diversity, evenness, or number of species per sampling date occurred. Normal classification analysis indicated seasonal clustering of samples collected over a 19 month period. Clusters, though exhibiting overlapping elements between these groups, show repeatable yearly trends characteristic of habitats in a dynamic "equilibrium," not successional state. The majority of benthic species at Barnstable Harbor exhibit distinctive seasonal fluctuations in numerical abundance, and most appear to be rather short-lived (1–3 years). Due to the dynamic temporal nature of recruitment and mortality patterns characteristic of short-lived species, it is not surprising that one finds seasonal species specific population trends at station 1. Inverse classification analysis clustered species into four groups, generally corresponding to seasonal population abundance peaks and periods of reproductive activity.

The majority of studies examining mechanisms influencing seasonal abundance patterns of species attribute such change to regular or systematic change in resource quality and abundance (Fretwell, 1972). Since the benthic fauna inhabiting the intertidal sand and mud flats are greatly affected by sedimentary characteristics, seasonal changes in sediment could promote population change. As mentioned, however, there were no significant changes in sediment size composition over a 19 month period. Thorson (1957) and Muus (1967) have suggested that settling patterns of many benthic organisms coincide with food availability. Seasonal fluctuations in planktonic organisms are common in this area of New England (Lillick, 1937) and could affect benthic organisms feeding upon them. However, most of the species occurring at Barnstable Harbor are infaunal deposit-feeders which obtain the majority of their nutrition directly from sediments. Temporal change in sediment food supply (measured as total organic carbon and potential food particulate matter) remained relatively stable throughout the year at most of the sampling stations (Whitlatch, in preparation). The effects of well-buffered seasonal food supplies influencing population characteristics of deposit-feeders has been reviewed by Levinton (1972).

Temperature is commonly considered to be the most important single factor influencing the distribution and reproductive activities of marine invertebrates (Thorson, 1946). Temperature fluctuations at Barnstable Harbor show marked temporal variation and are repeatable from year to year; two features that could affect observed repeatable seasonal clustering patterns of species groups. While temperature may provide a seasonal cue for community change, it does not totally explain why some groups of organisms are commonly found in the winter months while others are restricted to the summer months.

A partial explanation of the occurrence of seasonal species groups can be obtained from a closer examination of the latitudinal distributional patterns of many of the species. Cape Cod, Massachusetts, has long been considered a zoogeographic boundary between the southern part of the cold-temperate Nova Scotian faunal province and the northern portion of the mild-temperate Virginian faunal province (see Hazel, 1970, for a historical review) due to differences in summer water temperature found on the north and south sides of the Cape (Schopf, 1967). Several studies of offshore benthic organisms have shown distinct faunal elements characteristic of these two areas (Parker, 1948; Peterson, 1964; Schopf, 1965; Hazel, 1970). Barnstable Harbor is somewhat unique in that it contains faunal elements common to both zoogeographic provinces, coupled with a group of species found to occur throughout the entire eastern coast of North America.

Distributional ranges of the various species, summarized in Table III, indicate several distinct trends when related to seasonal temperature fluctuations. The Virginian components are generally found at their greatest abundance during the warmest months of the year (August–October), while the Nova Scotian elements are most common during the coldest temperature months of the year (January–April). The cosmopolitan forms, comprising the largest group of species, generally are most abundant during early spring and summer months. The Nova Scotian and Virginian faunal elements do not seem to be geographically limited by winter temperature since shallow water temperatures are about the same on both sides of the Cape (Schroeder, 1966; Schopf, 1967). Potentially southward migrating forms are limited by summer temperatures too high for adult survival. Northward migrating species are limited by summer temperatures that never are high enough for reproductive activity to be initiated. Summer water temperatures at Barnstable Harbor are higher than surrounding Cape Cod Bay water due to the shallow nature of the area, explaining the occurrence of Virginian faunal components.

While the examination of faunal elements characteristic of the two zoogeographic provinces helped to shed light on mechanisms influencing some seasonal community change, other species may be responding to the effects of biological interactions (mainly competition and predation) from other species. Certain species of deposit-feeding polychaetes which overlap greatly in both food and space utilization are temporally segregated, thus reducing the possibility of competitive interactions in resource use (Whitlatch, in preparation). Also, common epifaunal predators at Barnstable Harbor (mainly *Fundulus heteroclitus* and *Nassarius obsoletus*) which are abundant during the summer-fall months, can exert disproportionate seasonal effects on infaunal populations and can alter the species dominance structure of soft-bottom communities (Schneider, 1976; Whitlatch, unpublished). Species reproducing during times of minimal predator activity could significantly reduce the effects of predation.

The degree to which seasonal clustering patterns at the various habitats in Barnstable Harbor persist for longer periods of time is unknown at present. Comparisons of the present study with other studies of Barnstable Harbor (Sanders, *et al.,* 1962; Mills, 1967) revealed major long-term changes in both sedimentary features and species structure at several localities during the summer months. While temperature appears to be one of the controlling mechanisms for initiating reproductive activity in most species, the exact timing of the appearance of larvae in benthic populations certainly is influenced by year-to-year fluctuations in temperature cycles characteristic of this area of New England. This unpredictable environmental variable will place constraints on the degree of long-term stability of the re-occurring seasonal species groups at Barnstable Harbor. Lie and Evans (1973) provide one of the few studies examining long-term (7 years) changes in species composition of various faunal assemblages in subtidal areas of Puget Sound, Washington. While they found relatively small species changes, Lie (1968) indicated that there was little fluctuation in environmental parameters (*e.g.,* temperature) in this area—a feature unlike Barnstable Harbor.

Seasonal changes in community structure have been noted in several subtidal surveys (*e.g.,* Tenore, 1972; Boesch, 1973) and several authors have recorded seasonal groups of particular organisms in subtidal areas (Levings, 1975; Muus, 1967; Bodiou and Chardy, 1973; Grassle and Smith, 1976). Environments characteristic of seasonal patterns of species composition are relatively unstable and "physically-controlled" (Sanders, 1968); namely, estuarine or intertidal and shallow-subtidal habitats where both the probability of environmental disturbance and magnitude of seasonal fluctuations in environmental parameters are more likely to affect biological changes. The distinct seasonal clusterings of species at Barnstable Harbor, while an important characteristic of the area, may be even more pronounced than other areas owing to its somewhat unique geographic position and hydrographic conditions which have the potential of magnifying the effects of temporal community change.

In a review of the community concept and its validity when applied to marine benthic ecology, Stephenson (1973) pointed out the lack of the addition of a *temporal* component to most community definitions. While most benthic ecologists view communities as loosely organized suites of re-occurring species, they fail to recognize the dynamic temporal nature of the organization of the component members. This failure can be largely attributed to the short-term ("one-shot") nature of sampling in the vast majority of sampling programs. To better understand the nature of communities, physical and biological parameters which influence them, and their persistence or stability, it is important to gain knowledge of temporal changes in community structure. Understanding these patterns will provide further insight into the nature of the structure and evolution of marine soft-bottom biological systems.

This paper forms a portion of a thesis submitted to the University of Chicago in partial fulfillment of the requirements for a Ph.D. degree. I would like to dedicate this work to Ralph G. Johnson, friend and advisor, whose significant contributions in the field of marine benthic ecology have been a constant source of personal stimulation. Dr. H. L. Sanders and J. F. Grassle provided important insight into various aspects of the study. Dr. Grassle provided use of the classification program. I wish to thank the assistance of Ty Ranta, Barnstable Conservation Department for unlimited access to the marsh and use of a boat for collecting samples. L. F. Boyer, G. Johnson, and A. Williams aided with field work. Grants from Sigma Xi and the Hinds Fund for Evolutionary Research, University of Chicago, helped in starting the project. This research was largely supported by NSF grant DES 72–01608–A01 and an equipment grant from the Louis B. Block Fund made to R. G. Johnson, University of Chicago.

SUMMARY

1. A quantitative sampling survey of the benthic macrofauna inhabiting the intertidal sand and mud flats of Barnstable Harbor, Massachusetts, was conducted to describe general community structure and examine temporal changes in species composition.

2. Classification analysis delimited coarse and fine sand, mud, muddy-sand, and gravel-mud benthic species associations. The 32 species used in the inverse classification analysis were partitioned into 10 species groups, reflecting spatial distributional patterns. Many of the species were both dominant and ubiquitous, masking discrete species groupings.

3. The majority of macrobenthos at Barnstable Harbor were deposit-feeders which comprised more than 90% of all organisms sampled. The deposit-feeders normally dominate mud and muddy-sand sediments. Suspension-feeders were most abundant in fine sands. The relationship of sedimentary parameters affecting the distribution of both trophic groups proposed by Sanders is generally supported.

4. While no significant changes were evident in species diversity, evenness, or species number throughout a 19 month sampling period, classification analysis delimited seasonal clustering of both samples and species groupings. These patterns were repeatable over a two year period suggesting that dynamic "equilibrium," not successional change was occurring.

5. Seasonal clustering patterns of some species were related to the appearance of different zoogeographic faunal province elements. Typical warm-temperature Virginian components and cold-temperate Nova Scotian species were commonly found in late summer and winter, respectively.

LITERATURE CITED

Abbott, R. T., 1954. *American seashells.* D. Van Nostrand, Co., Princeton, 541 pp.

Ayers, J., 1959. The hydrography of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.,* 4: 448-462.

Barnes, R. D., 1974. *Invertebrate zoology.* W. B. Saunders, Co., Philadelphia, 870 pp.

Bloom, S. A., J. L. Simon, and V. D. Hunter, 1972. Animal-sediment relations and community analysis of a Florida estuary. *Mar. Biol.,* **13**: 43–56.

Bodiou, J.-Y., and P. Chardy, 1973. Analyse en composantes principales du cycle annuel d'un peuplement de copepods harpactoides de sables fin infralittoraux de Banyuls-sur-Mer. *Mar. Biol.,* **20**: 27–34.

Boesch, D. F., 1973. Classification and community structure of macrobenthos in the Hampton Roads area, Virginia. *Mar. Biol.,* **21**: 226–244.

Bousfield, E. L., 1973. *Shallow-water gammaridean Amphipoda of New England.* Cornell University Press, Ithaca, 312 pp.

Bradley, W. H., and R. Cooke, 1959. Living and ancient populations of the clam *Gemma gemma* in a Maine coast tidal flat. *U. S. Fish. Wildl. Serv. Fish. Bull.,* **58**: 304–334.

Buchanan, J. B., and R. M. Warwick, 1974. An estimation of benthic macrofauna production in the offshore mud of the Northumberland coast. *J. Mar. Biol. Assoc. U. K.,* **54**: 304–334.

Day, J. H., 1973. New polychaetes from Beaufort, with a key to all species recorded from North Carolina. *National Oceanic and Atmospheric Administration, Technical Report,* **375**: 1–140.

Dean, D., 1965. On the reproduction and larval development of *Streblospio benedicti* Webster. *Biol. Bull.,* **128**: 67–76.

Folk, R. L., 1966. A review of grain-size parameters. *Sedimentology,* **6**: 3–26.

Foster, N. M., 1971. Spionidae (Polychaeta) of the Gulf of Mexico and the Caribbean Sea. *Fauna Curacao Other Carib. Islands,* **36**: 1–183.

Fretwell, S. D., 1972. *Populations in a seasonal environment.* Princeton University Press, Princeton, 317 pp.

George, J. D., 1966. Reproduction and early development of the spionid polychaete *Scolecolepides viridis* Verrill. *Biol. Bull.,* **130**: 76–93.

Grant, D. C., 1965. Specific diversity in the infauna of an intertidal sand community. *Ph.D. Dissertation, Yale University,* New Haven, Connecticut, 53 pp.

Grassle, J. F., and J. P. Grassle, 1974. Opportunistic life histories and genetic systems in marine benthic polychaetes. *J. Mar. Res.,* **32**: 253–284.

Grassle, J. F., and W. Smith, 1976. A similarity measure sensitive to the contribution of rare species and its use in investigation of variation in marine benthic communities. *Oecologia,* **25**: 13–22.

Green, R. H., and K. D. Hobson, 1970. Spatial and temporal structure in a temperate intertidal community, with special emphasis on *Gemma gemma* (Pelecypoda: Mollusca). *Ecology,* **51**: 999–1011.

Hazel, J. E., 1970. Atlantic continental shelf and slope of the United States—Ostracode zoogeography in the southern Nova Scotia and northern Virginian faunal provinces. *U. S. Geol. Sur. Prof. Pap.,* **259E**: 1–22.

Holland, A. F., and T. T. Polgar, 1976. Seasonal changes in the structure of an intertidal community. *Mar. Biol.,* **37**: 341–348.

Hughes, R. N., and M. L. H. Thomas, 1971. The classification and ordination of shallow-water benthic samples from Prince Edward Island, Canada. *J. Exp. Mar. Biol. Ecol.,* 7: 1–39.

Johnson, R. G., 1970. Variations in diversity within benthic marine communities. *Am. Natur.,* 104: 285–300.

Jones, M. L., 1961. A quantitative evaluation of the benthic fauna off Point Richmond, California. *Univ. Calif. Publ. Zool.*, **67**: 219-320.

Lance, G. N., and W. T. Williams, 1967. A general theory of classificatory sorting strategies. I. Hierarchial systems. *Comput. J.*, **9**: 373–380.

Levings, C. D., 1975. Analysis of temporal variation in the structure of a shallow-water benthic community in Nova Scotia. *Int. Rev. Ges. Hydrobiol.*, **55**: 449–469.

Levinton, J., 1972. Stability and trophic structure in deposit-feeding and suspension-feeding communities. *Am. Natur.*, **106**: 472–486.

Lie, U., 1968. A quantitative study of benthic infauna in Puget Sound, Washington, U. S. A., in 1963–1964. *Fiskeridir. Skr. Ser. Havunders.*, **14**: 229–556.

Lie, U., and R. A. Evans, 1973. Long-term variability in the structure of subtidal benthic communities in Puget Sound, Washington, U. S. A. *Mar. Biol.*, **21**: 122–126.

Lie, U., and J. C. Kelley, 1970. Benthic infauna communities off the coast of Washington and in Puget Sound: identification and distribution of the communities. *J. Fish. Res. Board Can.*, **27**: 621–651.

Lillick, L., 1937. Seasonal studies of the phytoplankton off Woods Hole, Massachusetts. *Biol. Bull.*, **73**: 488–503.

McNulty, J. K., R. C. Work, and H. B. Moore, 1962. Some relationships between the infauna of the level bottom and the sediment in South Florida. *Bull. Mar. Sci.*, **12**: 322–332.

Mills, E. L., 1967. The biology of an Ampeliscid amphipod crustacean sibling species pair. *J. Fish. Res. Board Can.*, **24**: 305–355.

Morgans, J. F. C., 1956. Notes on the analysis of shallow water soft substrata. *J. Anim. Ecol.*, **25**: 367–387.

Muus, B. J., 1967. The fauna of Danish estuaries and lagoons. Distribution and ecology of dominating species in the shallow beaches of the mesohaline zone. *Medd. Dan. Fisk. Havunders.* **5**: 1–316.

Parker, F. W., 1948. Foraminifera of the continental shelf from the Gulf of Maine to Maryland. *Harvard Univ. Mus. Comp. Zool. Bull.*, **100**: 213-241.

Pearson, T. H., 1971. The benthic ecology of Loch Linnhe and Loch Eil, a sea-loch system on the west coast of Scotland. III. The effect on the benthic fauna of the introduction of pulp mill effluent. *J. Exp. Mar. Biol. Ecol.*, **6**: 211–233.

Peterson, C. G. J., 1918. The sea bottom and its production of fish food. A survey of the work done in connection with the valuation of the Danish waters from 1883–1917. *Rep. Dan. Biol. Sta.*, **25**: 1–62.

Peterson, K. W., 1964. Some preliminary results of a taxonomic study of the Hydrozoa of the Cape Cod area. *Biol. Bull.*, **127**: 384–385.

Pettibone, M. H., 1963. *Marine polychaete worms of the New England region. 1. Aphroditidae through Trochochaetidae.* Smithsonian Institution Museum of Natural History, Washington, D. C., 356 pp.

Pielou, E. C., 1966. The measurement of diversity in different types of biological collections. *J. Theor. Biol.*, **13**: 131–144.

Redfield, A. C., 1972. Development of a New England salt marsh. *Ecol. Monogr.*, **42**: 201–237.

Sanders, H. L., 1958. Benthic studies in Buzzards Bay. I. Animal-sediment relationships. *Limnol. Oceanogr.*, **3**: 245–258.

Sanders, H. L., 1960. Benthic studies in Buzzards Bay. III. The structure of the soft-bottom community. *Limnol. Oceanogr.*, **5**: 138–153.

Sanders, H. L., 1968. Marine benthic diversity: a comparative study. *Am. Natur.*, **102**: 243–282.

Sanders, H. L., E. M. Goudsmit, E. L. Mills, and G. R. Hampson, 1962. A study of the intertidal fauna of Barnstable Harbor, Massachusetts. *Limnol. Oceanogr.*, **7**: 63–79.

Scheltema, R. S., 1962. Pelagic larvae of New England intertidal gastropods. I. *Nassarius obsoletus* and *Nassarius vibex*. *Trans. Am. Microsc. Soc.*, **8**: 1–12.

Schneider, D. C., 1976. The structural reorganization of a salt marsh community following a disturbance. *Master's Thesis, State University of New York*, Stony Brook, New York, 48 pp.

SCHOPF, T. J. M., 1965. Ectoproct (Bryozoa) distribution in the Atlantic continental shelf from Hudson Canyon to Nova Scotia. *Biol. Bull.,* **129**: 421–422.

SCHOPF, T. J. M., 1967. Bottom-water temperatures on the continental shelf off New England. *U. S. Geol. Sur. Prof. Pap.,* **575D**: 192–197.

SCHROEDER, E. H., 1966. Average surface temperatures of the western North Atlantic. *Bull. Mar. Sci.,* **16**: 302–323.

SHANNON, C. E., AND W. WEAVER, 1963. *The mathematical theory of communication.* University of Illinois Press, Urbana, 117 pp.

SIMON, J. L., 1967. Reproduction and larval development of *Spio setosa* (Spionidae; Polychaeta). *Bull. Mar. Sci.,* **17**: 398–431.

SIMON, J. L., 1968. Occurrence of pelagic larvae in *Spio setosa* Verrill, 1873 (Polychaeta: Spionidae). *Biol. Bull.,* **134**: 503–515.

SIMON, J. L., AND K. M. BRANDER, 1967. Reproductive biology and larval systematics of Cape Cod polychaetous annelids. Pages 41–44 in *Systematics-ecology fifth annual report.* Marine Biological Laboratory, Woods Hole, Massachusetts.

STEPHENSON, W., 1973. The validity of the community concept in marine biology. *Proc. R. Soc. Queensl.,* **84**: 73–86.

STEPHENSON, W., AND G. N. LANCE, 1970. The macrobenthos of Moreton Bay. *Ecol. Monogr.,* **40**: 459–494.

STEPHENSON, W., W. T. WILLIAMS, AND S. COOK, 1972. Computer analysis of Peterson's original data on bottom communities. *Ecol. Monogr.,* **42**: 387–415.

TENORE, K. R., 1972. Macrobenthos of the Pamlico River estuary, North Carolina. *Ecol. Mongr.,* **42**: 51–69.

THORSON, G., 1946. Reproduction and larval development of Danish marine bottom invertebrates. *Medd. Komm. Fisk. Havunders. Copenhagen Ser. Plankton,* **4**: 1–523.

THORSON, G., 1957. Bottom communities (sublittoral or shallow shelf). Pages 461–534, in J. W. Hedgpeth, Ed., *Treatise on marine ecology and paleoecology, Volume 1.* Geological Society of America Memoir 67, Waverly Press, Baltimore, 1296 pp.

TURNER, H. J., 1951. *Fourth report on investigation of the shellfisheries of Massachusetts.* Commonwealth of Massachusetts, Department of Conservation, Division of Marine Fisheries, Boston, Massachusetts, 21 pp.

WATLING, L., 1975. Analysis of structural variations in a shallow water estuarine deposit-feeding community. *J. Exp. Mar. Biol. Ecol.,* **19**: 275–313.

WILLIAMS, W. T., 1971. Principles of clustering. *Annu. Rev. Ecol. Syst.,* **2**: 303–326.

Reference: *Biol. Bull.,* **152**: 295–310. (April, 1977)

CHARACTERISTICS OF INFLUX AND NET FLUX OF AMINO ACIDS IN *MYTILUS CALIFORNIANUS*

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Influx of ^{14}C-labelled amino acids across the epidermis has been observed in a number of soft-bodied marine invertebrates (see Stephens, 1972, for a review). Such influx in molluscs has been reported by several workers (recent examples include Anderson and Bedford, 1973; Bamford and Campbell, 1976; Bamford and McCrea, 1975; Péquignat, 1973; Stewart and Bamford, 1975; Wright, Johnson and Crowe, 1975). Some of these studies employed intact organisms, while others reported influx of amino acids into isolated gills or gill discs. In no case was net flux examined. The only report of net influx of amino acids in a mollusc is that of Stephens and Schinske (1961). They employed high external concentrations for most of their work (2 mM) but observed net influx in the clam, *Spisula,* from ambient glycine concentrations of 20 μM. However, they did not attempt to follow net influx closely and describe its kinetics, nor did they compare net flux with influx.

Simultaneous measurement of influx and net flux in lamellibranchs is of considerable interest for at least three reasons. First, it is net flux which is of interest in assessing the potential nutritional significance of trans-epidermal amino acid uptake for the organism. Several of the workers cited draw tentative conclusions based on the more or less clearly stated assumption that influx (their observed phenomenon) can be equated (sometimes with stated reservations) with net flux. This assumption may be approximately correct for sediment-dwelling forms. Stephens (1975) reports net influx of amino acids at ambient levels well below those found in the sediment environment. However, ambient concentrations of amino acids normally present in the water column are substantially lower. Hence influx cannot be assumed to reflect net flux at naturally occurring ambient concentrations for ciliary-mucoid filter feeders such as lamellibranchs.

The second reason for interest in net flux relates to studies of the mechanism of influx. Intracellular free amino acid pools are very large in marine invertebrates (typically of the order of $2–5 \times 10^{-1}$ M). In typical studies of influx, labelled amino acids are supplied at low ambient concentrations. Under such circumstances, if the labelled substrate were entering the organism by a process such as exchange diffusion, rapid influx of labelled material would be observed, even though accompanied by no flux or even by net efflux. Indeed, Johannes, Coward and Webb (1969) have proposed precisely this explanation to account for observations of influx of labelled amino acids, based on their studies of influx and net flux in an ectoparasitic flatworm, *Bdelloura.* They did not directly examine the predictable consequences of an exchange diffusion mechanism. However, this can readily be done if influx and net flux are monitored simultaneously under a suitable range of conditions.

Thirdly, Stewart and Bamford (1975) and Wright (1976) report evidence that influx of amino acids in molluscs is sodium-sensitive. Sodium dependence has yet to be demonstrated for transepidermal amino acid transport in any marine invertebrate, including molluscs. One of the key observations which is considered to be convincing evidence of sodium-coupled or sodium-dependent transport of an organic solute is demonstration of sodium-coupled efflux from cell to medium (Schultz and Curran, 1970). Simultaneous studies of influx and net flux as a function of external sodium permit such observations.

Studies of net flux of amino acids have been facilitated by the recent introduction of the reagent, fluorescamine (Udenfriend, Stein, Böhlen, Dairman, Leimgruber and Weigle, 1972). Fluorescamine forms a fluorescent product with primary amines in aqueous solution permitting simple and routine determination of amino acid concentrations as low as 10^{-7} M. North (1975) and Stephens (1975) describe procedures for determining primary amines in sea water using this reagent.

In the present report, the initial report of Wright *et al.* (1975) on the kinetics of amino acid influx in isolated gills of *Mytilus* is extended and refined. Simultaneous measurements of influx and net flux in this organism are also reported, and the implications of such measurements for hypotheses concerning the nutritional significance of amino acid influx and the mechanism of this influx are described.

MATERIALS AND METHODS

Specimens of *Mytilus californianus* were collected from the open coast of southern California near Laguna Beach. Animals were kept at 13° C in aquaria in aerated sea water. Preliminary studies showed no difference in experimental results between freshly collected mussels and those maintained in aquaria for eight weeks. Animals were usually used within four weeks of collection.

All experiments were conducted in artificial sea water (ASW, MBL) prepared with reagent grade salts and distilled water according to Cavanaugh (1956). When sodium-free incubation media were used, choline chloride or LiCl were substituted for NaCl, and $KHCO_3$ for $NaHCO_3$. These solutions were isosmotic to ASW, MBL (salinity = 31‰), and they had a pH of 7.8–8.0.

Mussels were opened by cutting the posterior adductor muscle. The four demibranchs were removed, placed on a sheet of aluminum foil and discs of gill tissue cut with a 1.0 cm stainless steel cork borer. These discs of gill tissue had a mean wet weight of 32.2 ± 0.7 (s.e.) mg, and a mean dry weight of 5.0 ± 0.1 mg. Gill discs were held in MBL at 16° C for thirty minutes prior to observations. Discs from at least three different mussels were used in all influx experiments. The procedure for determination of influx rates was as follows. Nine discs of gill tissue were introduced into 200 ml of aerated MBL containing cycloleucine (1-aminocyclopentane-1-carboxylic acid) or glycine. Solutions contained approximately 0.05 μCi/ml of (U-^{14}C)cycloleucine (New England Nuclear) or (U-^{14}C) glycine (Schwartz-Mann) and sufficient ^{12}C substrate to give final concentrations ranging from 2 μM to 5 mM. Three discs of tissue were removed from the solution at 3, 6 and 9 minutes, rinsed rapidly (5–10 seconds) in two successive 0° C MBL rinses, and blotted gently on filter paper. Individual discs were placed in scintillation vials containing 1 ml of 0.1 N HNO_3. After extraction for at least twelve hours, a toluene-based scintillation cocktail was added and the extracted discs were

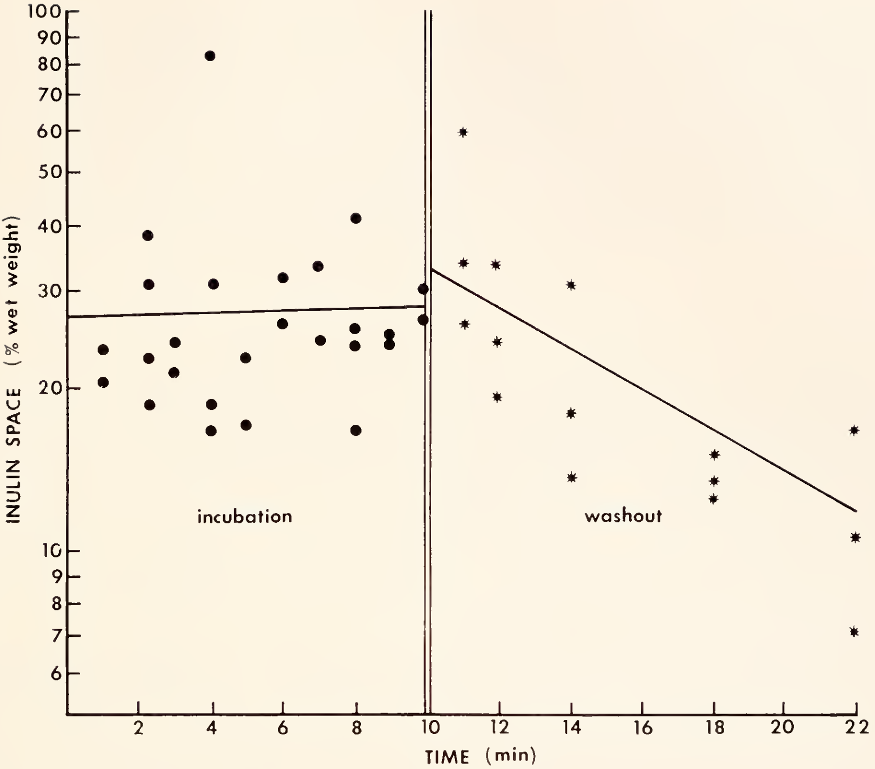

FIGURE 1. Labelling and washout of gill discs with ^{14}C-inulin. Labelling is expressed as inulin space in units of percentage of tissue wet weight. Each point represents radioactivity associated with a single gill disc after rinsing and blotting. Lines are least squares regressions.

counted using a Beckman CPM-100 scintillation counter. Initial and final samples of the medium were taken and counted for each experiment. The volume of the medium was sufficient so that no significant decrease in radioactivity was observed.

Extracellular space was determined using (U-^{14}C)inulin. A solution containing trace amounts of labelled inulin in MBL was prepared. After exposure to this solution for various times, sets of tissue discs were rinsed, blotted and extracted as described above. To determine the time course of washout of inulin, tissue discs were incubated in the labelled inulin solution for ten minutes, transferred to a large volume of MBL and removed at intervals. Radioactivity was determined as described.

Simultaneous measurements of influx and net flux of glycine in intact mussels were performed as follows. Mussels were cleaned with a wire brush and kept at 16° C in MBL containing streptomycin (200 mg/liter), chloramphenicol (100 mg/liter) and penicillin (500,000 I.U./liter) to reduce microbial activity. After 24 hours, animals were rinsed in MBL and placed individually in 500 ml of MBL containing 0.05 μCi/ml ^{14}C-glycine plus unlabelled substrate. Samples of the medium were taken at intervals after introducing the animal. One set of samples was counted for radioactivity. Another set was used for determination of primary amines as follows. A 0.5 ml sample was buffered to pH 9.2 using 0.5 ml of 0.2 M borate buffer and mixed vigorously with 0.5 ml of acetone containing fluorescamine (200 mg/liter). After addition of 2.0 ml of distilled water, fluorescence at 480 nm with excitation at 390 nm was measured using a Turner fluorometer (filters were used to approximate excitation and emission frequencies). A set of glycine standards was measured with each set of experimental determinations. Primary amine concentrations are expressed in glycine-equivalent concentration units.

RESULTS

Inulin space

When gill discs are exposed to ^{14}C-inulin, labelling is complete at the end of one minute and remains constant thereafter at approximately 30% of the wet weight of the tissue. This inulin space is interpreted to include rapidly labelled extracellular space together with the portion of the medium adhering to the gill discs after rinsing and blotting. When discs labelled with ^{14}C-inulin are placed in a large volume of MBL, radioactivity disappears exponentially with a half time of approximately seven minutes (Fig. 1). The exponential character of the washout is consistent with the hypothesis that adherent medium and extracellular tissue space behave as a single compartment.

The influence of the presence of this rapidly labelled extracellular compartment on determinations of influx rates of organic substrates is illustrated in Figure 2. In the upper portion of Figure 2, the results of exposure of gill discs to ^{14}C-cycloleucine at an ambient concentration of 50 μM is presented. Uptake is linear with time. A least squares regression line through the observed points intercepts the y-axis close to the point predicted by the inulin space. At this low concentration, the labelling of the inulin space (*i.e.,* ^{14}C-cycloleucine counted as a result of its presence in the extracellular space) contributes little to the total tissue radioactivity. It represents about 3% of the total radioactivity associated with the tissue after a ten-minute exposure to the solution. Washout of radioacivity when the tissue is placed in MBL is imperceptible.

The lower portion of Figure 2 presents similar observations, but cycloleucine is supplied at an ambient concentration of 5 mM. Rapid labelling of the inulin space accounts for a very significant fraction of the total radioactivity associated with the tissue. Again, uptake is linear and again a regression line intercepts the y-axis at the point predicted by measurement of the rapidly labelled extracellular compartment. However, although influx is more rapid at the higher ambient concentration, the carrier mediating influx is fully saturated and the total influx during the brief observation period is a small fraction of ambient concentration. Therefore, radioactivity associated with the rapidly labelled compartment accounts for approximately 66% of the total radioactivity present in the tissue at the end of ten minutes. The fact that a large fraction of the radioactivity was present in the extracellular space is also indicated by the exponential washout shown in the lower portion of Figure 2. If the rate of cycloleucine influx were calculated from the total amount of radioactivity associated with the tissue after a five-minute incubation, the rate would be overestimated by approximately 350%.

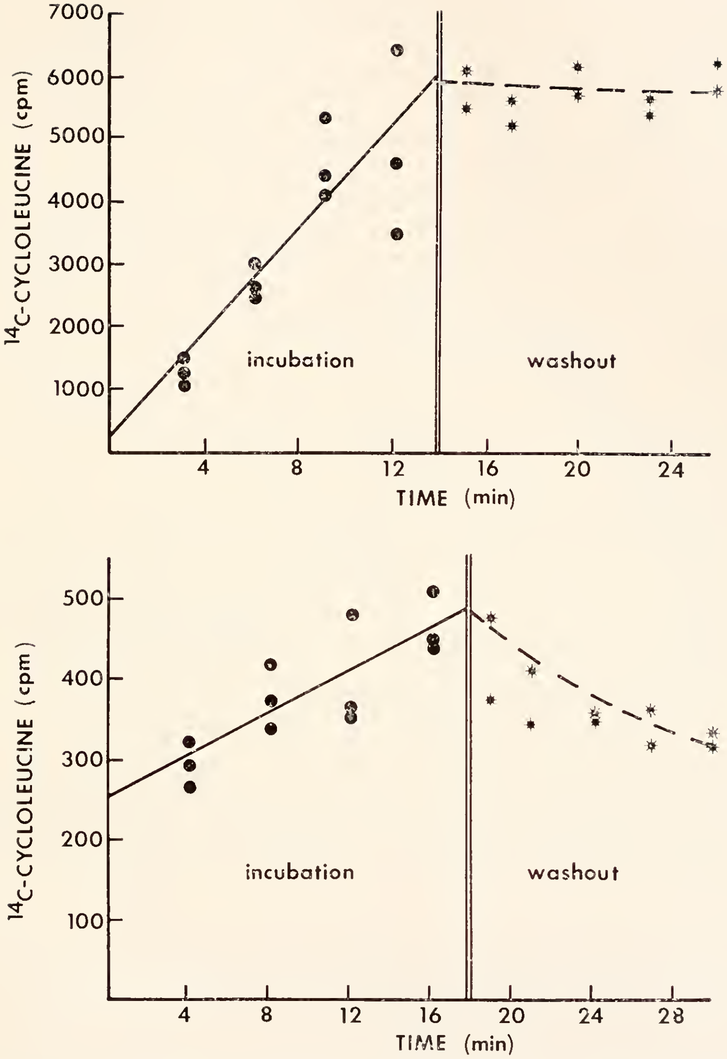

FIGURE 2. The time course of uptake and washout of ^{14}C-cycloleucine into gill discs incubated in two different concentrations of ^{12}C-cycloleucine. Each point is the radioactivity from a single gill disc. The solid lines were fit by the method of least squares; the dashed lines are the rates of washout predicted by the data in Figure 1. The y-intercepts predicted by the data in Figure 1 are 198 cpm and 255 cpm for the upper and lower graphs, respectively.

Kinetics of cycloleucine and glycine influx

The effect of increasing concentration of cycloleucine in the medium on influx of cycloleucine is shown in Figure 3. At low concentrations the influx of cycloleucine (J^i) increases in a nonlinear fashion with increasing concentration in the medium (S). At high concentrations, J^i increases linearly with S. However,

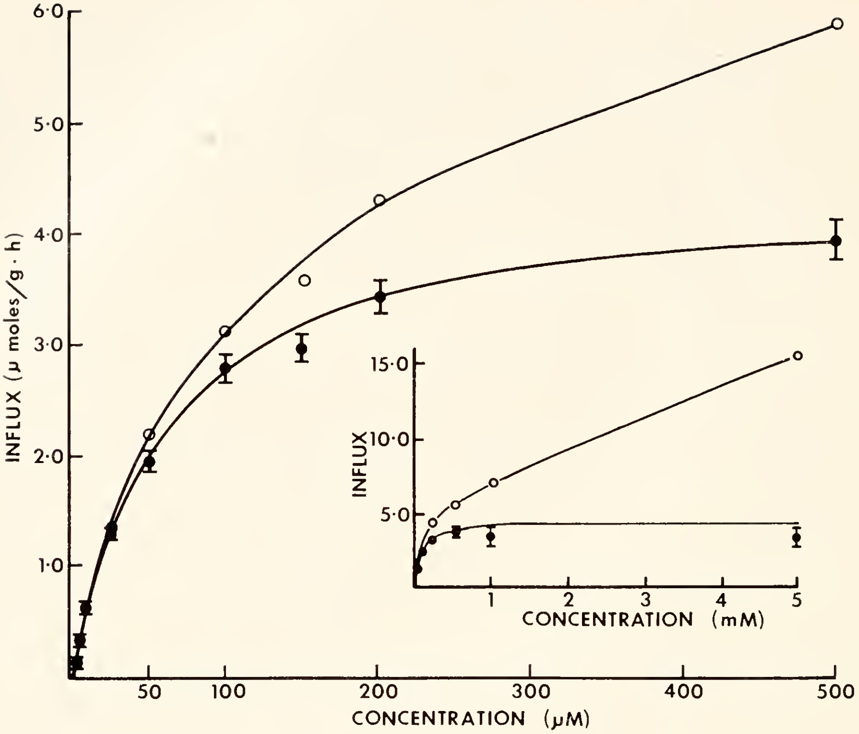

FIGURE 3. The influx rate of ^{14}C-cycloleucine into gill tissue as a function of increasing concentration of cycloleucine. Open circles are mean values for cycloleucine influx uncorrected for the presence of inulin space; closed circles are values for influx corrected for inulin space. Units of influx in the inset are µmoles/(g·hr); concentration is in mM. Bars signify ± 1 s.e. (n for each point ranges from 12 to 54).

when the data are corrected for ^{14}C-cycloleucine present in the rapidly labelled inulin space, this second linear component virtually disappears. The slope of J^i as a function of S at high concentrations is nearly zero. Cycloleucine influx based on corrected data is fully described by the Michaelis-Menten equation: $[J^i = (J^i_{max}(S))/(K_t + (S))]$, where J^i_{max} is the maximum rate of influx and K_t is the ambient concentration at which influx is half the maximum rate.

The constants J^i_{max} and K_t for cycloleucine influx were determined graphically from a plot of $(S)/J^i$ against (S). J^i_{max} is 4.5 µM/(g·hr) and K_t is 63 µM. Similar data were obtained for glycine influx. When corrected for the labelling of inulin space, the constants for glycine were determined to be $J^i_{max} = 4.6$ µM/(g·hr) and $K_t = 34$ µM. There was no residual "passive" component perceptible in the influx data for either cycloleucine or glycine after correcting for inulin space. J^i_{max} for the two substrates is not significantly different, suggesting that cycloleucine and glycine may enter by the same carrier-mediated pathway. This suggestion is also consistent with the inhibition data presented by Wright *et al.* (1975).

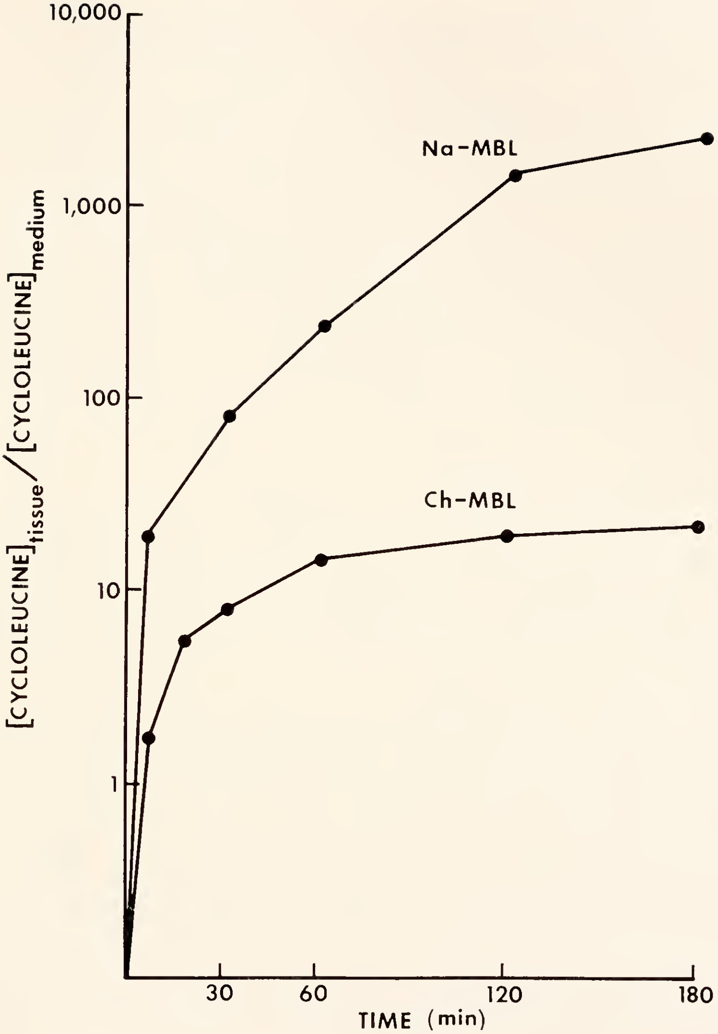

FIGURE 4. The tissue-to-medium concentration gradient of cycloleucine developed by isolated gill tissue in MBL (Na-MBL) or MBL with choline replacing sodium (Ch-MBL) as a function of time; at each point, n = 3.

Effect of external sodium on transport of cycloleucine

Influx of cycloleucine into gill discs was reduced to 10% or 20% of control values when sodium in the medium was replaced by lithium or choline respectively. Cycloleucine concentration was 50 μM. This inhibition of influx in the absence of sodium is at least partially reversible. When gill discs exposed to sodium-free MBL for one hour were washed in regular MBL for fifteen minutes, the rate of cycloleucine influx returned to 77% of control values.

Figure 4 presents data concerning steady state concentrations of cycloleucine present in gill tissue after prolonged exposure to a small volume of MBL containing 50 μM cycloleucine. Tissue concentration is calculated based on wet weight excluding inulin space. Since cycloleucine is not metabolized and is not normally present in *Mytilus*, concentration is simply related to radioactivity and specific activity. In the presence of sodium (425 mEq/liter = MBL) ambient cycloleucine decreases in our system (10 ml of medium) to approximately 10 μM, while tissue concentration increases over the course of three hours to approximately 20 mM. When sodium is replaced in the medium by choline, the medium concentration does not fall perceptibly below 50 μM while tissue concentration increases to approximately 1 mM. Similar observations using glycine as a substrate produce nominal concentration ratios based on radioactivity of approximately 15,000 : 1 in the presence of sodium; 10 : 1 in the absence of sodium. However, glycine is normally present in the intracellular free amino acid pool and is metabolized by the gill tissue so such figures cannot be interpreted simply.

Influx of glycine and net flux of primary amines

The following observations were made on intact mussels. Glycine was used as the substrate, both because it is a major constituent in naturally occurring free amino acids in sea water and because the specific fluorescence of the product of its reaction with fluorescamine is suitably high (North, 1975). Cycloleucine produces a weakly fluorescent product with fluorescamine so it was not suitable for these observations.

Figure 5 presents data for influx of ^{14}C-glycine based on disappearance of radioactivity from the medium together with data for net flux of primary amines based on the fluorescamine procedure. Samples of the medium were taken at the same time for the two procedures and various initial concentrations of glycine were employed as indicated in the figure. At high ambient concentrations (20–100 μM) influx of ^{14}C-glycine corresponds closely to net influx of primary amines. At the other extreme, primary amines appeared in the medium when mussels were placed in MBL containing no amino acid and reached a steady state concentration of 2–4 μM which was stable for at least 24 hours. At ambient concentrations of ^{14}C-glycine below 20 μM, influx of glycine estimated from radioactivity is consistently more rapid than net influx of primary amines estimated from fluorescamine determinations. This discrepancy becomes more pronounced as ^{14}C-glycine concentrations decrease.

This relation is expressed graphically in Figure 6. The ratio ^{14}C-glycine/primary amine is plotted against glycine concentration as estimated by radioactivity of the solution. At high concentrations that ratio approaches unity. At low concentrations, the efflux of the unknown primary amines becomes increasingly significant relative to ^{14}C-glycine influx and the ratio declines. Data in Figure 6 include those presented in Figure 5, as well as additional data from similar experiments. The line in Figure 6 is drawn to fit the equation $y = x/(x + 1.06)$, and represents the assumption that efflux is constant at all concentrations of glycine in the medium. The mean difference in concentration between ^{14}C-glycine and total primary amine is 1.06 μM in the ten cases observed where ^{14}C-glycine concentration was reduced to less than 10 μM.

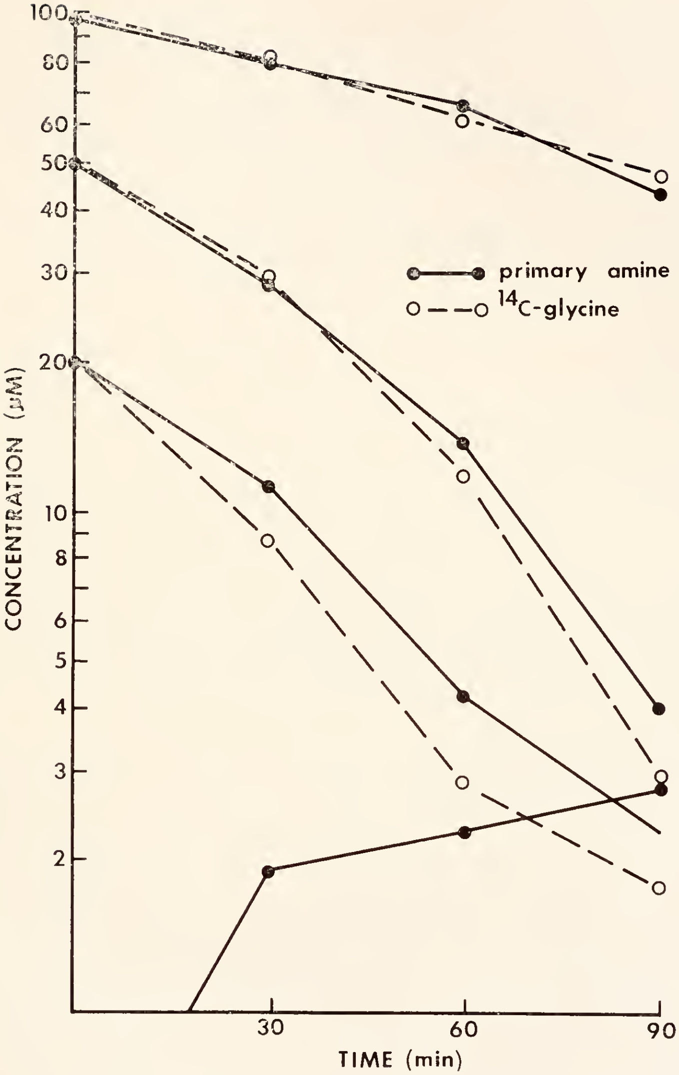

FIGURE 5. The clearance of ^{14}C-glycine and total primary amine from MBL by intact *Mytilus* as a function of time. The volume of experimental medium in each experiment was 500 ml, and the initial concentrations of glycine were 100 μM, 50, 20, and 0. The closed circles are the concentrations of total primary amine measured as glycine equivalents using fluorescamine. The open circles are the concentrations of ^{14}C-glycine. Each line represents the clearance by one mussel.

Influx of glycine and net flux of primary amines: sodium effects

Gill discs liberate primary amines into the medium resulting in a relatively high background of fluorescamine-positive material. This was a reason for choosing intact mussels for the preceding observations. However, the replacement of external

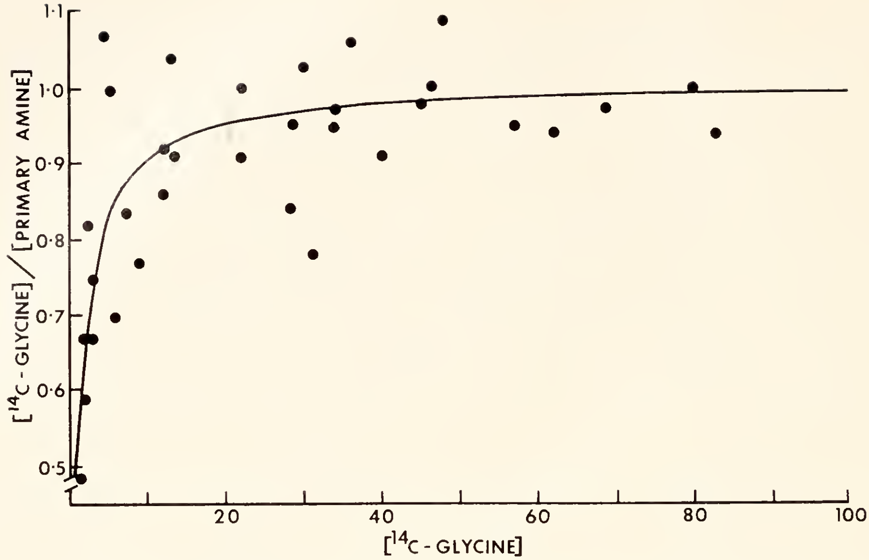

FIGURE 6. The ratio of the concentration in the medium of radioactive glycine to total primary amine measured in duplicate samples during clearance experiments plotted as a function of the radioactive glycine concentration. Data were taken from Figure 5 and similar experiments. The line fits the equation, $y = x/(x + 1.06)$, and expresses the assumption that efflux is constant at all concentrations of glycine in the medium.

sodium with other ions interferes with normal pumping activity of the lateral cilia. Therefore, it was necessary to use isolated discs of gill tissue to examine the influence of external sodium on the relation between influx and net flux.

Three discs were placed into each of a series of beakers containing 10 ml of MBL plus 10 μM ^{14}C-glycine. A parallel set of tissue discs were placed into beakers containing 10 ml of MBL in which sodium had been replaced by choline, also containing 10 μM ^{14}C-glycine. At prescribed times the tissue in a beaker was removed and samples of the medium taken. Figure 7 presents the results of following the level of radioactivity and the level of primary amines in such an experiment. Radioactivity in the MBL medium declined to very low levels in the course of the experiment (equivalent to about 0.2 μM at 4 hours); primary amines fluctuated between 8 and 16 μM expressed as glycine equivalents with no clear trend with time. When sodium was replaced with choline, radioactivity decreased very slightly over the four-hour period. Primary amines in the medium increased steadily reaching 37 μM as glycine equivalents at the end of four hours.

DISCUSSION

The data presented here show that the influx of cycloleucine and glycine is carrier-mediated. The *in vitro* gill preparation yields uptake data that are, however, easily misinterpreted as being indicative of a two component transport process: at low concentrations (< 20 μM), uptake appears to involve a high affinity saturable system, while at high concentrations (> 20 μM), either a low affinity system or passive diffusion appears to become increasingly important. However, when the data are corrected for the presence of the inulin space, influx appears to be by a single component, fully saturable system, which follows Michaelis-Menten type kinetics. No evidence was found to support postulation of a second, low-affinity carrier system. The close agreement of J^{i}_{max} for the two substrates suggests that glycine and cycloleucine enter *via* the same pathway(s).

Passive diffusion can play no significant role in the influx of amino acids. *Mytilus* and other bivalves maintain high steady-state concentrations of free amino acids in their tissues. If the gill membrane had permeability properties such that passive diffusion could occur from external concentrations in the 100 μM range

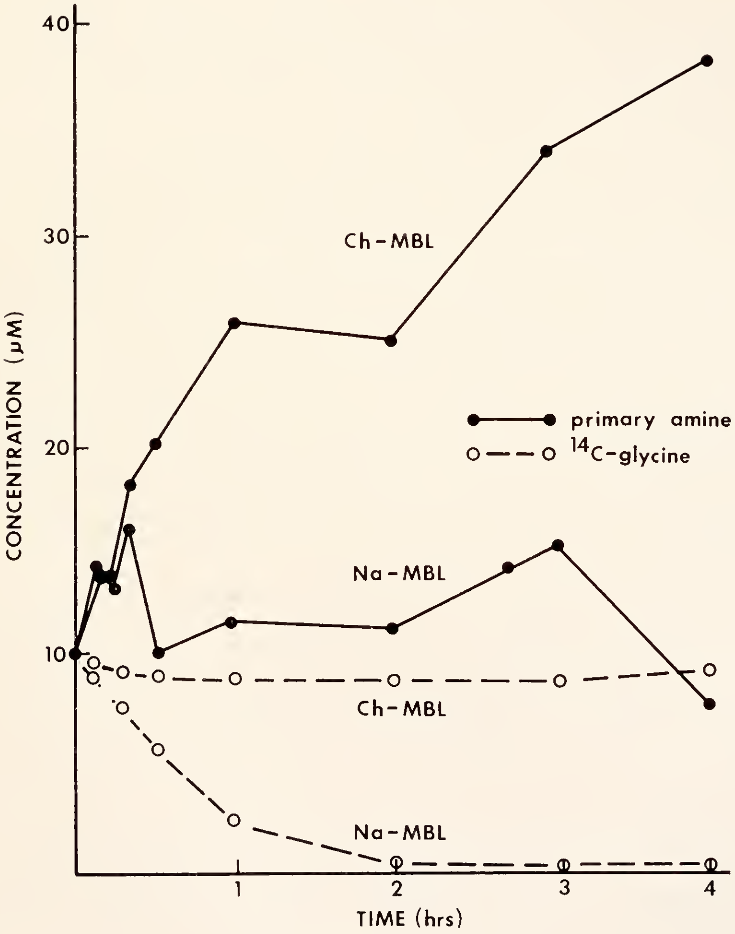

FIGURE 7. The change in medium concentration of ^{14}C-glycine and total primary amine resulting from the incubation of isolated gill tissue in either Na-MBL or Ch-MBL as a function of time. The data obtained at each time interval represent the measurements of medium concentration in separate parallel experiments. Duplicate samples were taken for the determination of radioactivity and total primary amine.

at rates falsely implied by the uncorrected data, the symmetrical passive efflux of amino acid from the approximately half molar intracellular free amino acid pool would make the maintenance of such pools energetically impossible.

The kinetic parameters calculated from the data on influx rates for cycloleucine and glycine are similar to the K_t's and V_{max}'s reported for uptake of several neutral amino acids by other preparations of isolated bivalve gill (Wright, *et al.*, 1975 on *Mytilus californianus;* Bamford and Campbell, 1976 on *M. edulis;* Bamford and McCrea, 1975 on *Cerastoderma edule;* Stewart and Bamford, 1975 on *Mya arenaria;* Anderson and Bedford, 1973 on *Rangia cuneata*). These investigators reported K_t's ranging from 12 μM to 250 μM, and V_{max}'s from approximately 0.5 μM/(g·hr) to 35 μM/(g·hr). However, none of the above workers corrected their data for the possible presence of a rapidly-labelled extracellular compartment such as the one noted in the present study. The importance of such a correction for the interpretation of uptake data makes the kinetic parameters of these carrier studies suspect. We wish to replace the values reported earlier (Wright, *et al.*, 1975) with those in the present work.

The data summarized in Figures 5 and 6 indicate that the disapparance of radioactivity from the medium as a result of glycine influx into *Mytilus* accurately represents net influx of glycine into the organism at concentrations greater than 20 μM. This does not exclude the possibility that glycine influx and the net influx observed may not be accompanied by efflux of small amounts of unidentified primary amines. Such an efflux is the simplest explanation of the discrepancy observed between glycine influx and net influx at lower ambient concentrations. If efflux also occurs at higher concentrations, it would be expected to elevate primary amine determinations by a few parts per hundred at the higher concentrations employed in this study; this is about the range of analytical error for the procedure.

The close parallel of influx and net flux at high concentrations excludes simple exchange diffusion as a mechanism to account for influx of ^{14}C-glycine. That hypothesis predicts a stable ambient concentration of primary amines at the initial level accompanied by an exponential decrease in radioactivity, which is contrary to the data presented here.

Primary amines in the medium are reduced by *Mytilus* to levels ranging from 2–5 μM (as glycine equivalents), the concentration range in which steady-state is achieved. The steady-state seems independent of the initial level of amino acid supplied. At concentrations greater than 2–5 μM, net influx is observed until steady-state is reached. At concentrations lower than this, steady-state is achieved by net efflux. It should be emphasized that the primary amines which appear in the medium are at present unidentified. Expression of their concentration as glycine-equivalents is arbitrary. The specific fluorescence of reaction products of primary amines with fluorescamine varies; as noted, cycloleucine produces a weakly fluorescent product; the products formed with polybasic amino acids fluoresce more strongly than glycine. The compound(s) which appear in the medium are currently being studied. With respect to the mechanism of efflux, simple exchange diffusion can be excluded, but further conclusions cannot be drawn.

These observations do not permit definite conclusions about the role of transepidermal amino acid uptake in the nutrition of *Mytilus*. If the animal experiences ambient concentrations greater than a few micromoles/liter in its environment, net influx seems likely. This is much higher than the levels reported for free amino acids in the water column which range from 0.2 to 2 micromolar. Therefore, mussels presumably encounter sea water passing across their gills which is lower in amino acid content than the steady-state ambient concentration reported here. Despite that fact, the possibility of net influx from amino acid concentrations in the micromolar range cannot be excluded on the basis of the present work. If no change in the composition of primary amine occurred in our closed system, the steady-state level observed would be a measure of the capacity of *Mytilus* to clear primary amines from micromolar concentrations. However, there is no reason to suspect that this is the case. In an open system either net influx or efflux could occur. The direction of net movement of amino acid depends on the ratio of the rate of acquisition of primary amine(s) (naturally occurring) to the loss of primary amines(s) (from the gill) in a single pass of incurrent water across the gills.

Influx of ^{14}C-glycine in gill discs of *Mytilus* is inhibited when external sodium is replaced by choline or lithium. This agrees with the observation reported by Stewart and Bamford (1975) of reduced rates of ^{14}C-alanine influx into the isolated gills of the soft-shelled clam, *Mya arenaria,* in the absence of external sodium. Sensitivity to external sodium has been reported for many nonelectrolyte transport systems (reviewed by Schultz and Curran, 1970). The observation of a relation between external sodium and the rate of influx for an organic solute is an initial step in establishing the occurrence of sodium-coupled cotransport, and is consistent with the presence of such a system. However, it must be accompanied by additional observations before a sodium-coupled cotransport mechanism can be considered to be established. This has not been done for transepidermal amino acid transport in any marine invertebrate.

The present report of efflux of primary amines when sodium is replaced in the medium (Fig. 7) is also consistent with the existence of sodium-coupled cotransport in *Mytilus* gill. One of the key predictions made by sodium-coupled models of transport of organic solutes is that the organic solute should be translocated from the cell in circumstances where internal sodium exceeds external sodium (Schultz and Curran, 1970). The primary amines which appear in the medium bathing gill discs when external sodium is replaced with choline have not been identified. Therefore detailed discussion is not justified. However, it can be noted that if the primary amines are interpreted as free amino acids, the rate of efflux shown in the data of Figure 7 is approximately 20% of the J^i_{max} for glycine and cycloleucine. This is an unreliable figure for an additional reason; sodium may affect J_{max}, and intracellular sodium has not been measured. Nevertheless, efflux under such conditions is consistent with a sodium-coupled transport system for amino acids.

Despite the support offered by the present observations for the existence of a sodium-coupled cotransport system, these data cast doubt on the ability of such a system to account fully for amino acid transport in the gill of *Mytilus.* First, there is apparent concentrative transport of cycloleucine in the absence of external sodium (Fig. 4). This is contrary to the prediction based on a sodium-coupled model. The data seem unambiguous, since cycloleucine is neither normally present in *Mytilus* nor is it metabolized. It is possible to frame an explanation based on hetero-exchange diffusion, with evidence for efflux into sodium-free medium. If this were coupled to an exchange entry of cycloleucine, apparent concentrative transport would be observed. However, evidence indicates exchange diffusion does not normally occur, and it would not be predicted for a sodium-coupled model in the absence of external sodium.

The second *caveat* concerning a sodium-coupled mechanism as a complete explanation for these data is based on thermodynamic considerations. The basic feature of mechanisms for transport of organic solutes coupled to sodium transport is the stipulation that at least part of the energy necessary for active transport of the organic solute is not supplied by direct metabolic energy coupling but by the sodium concentration difference between the external solution and cytoplasm, *via* a coupled transport of sodium and the organic solute. Jacquez and Schafer (1969) formulated a series of equations to compare energy expenditure available from the sodium electrochemical potential gradient. The model for the coupling of the fluxes of amino acid and sodium most often proposed in conjunction with the sodium-gradient hypothesis involves the electrogenic 1:1 cotransport of sodium and amino acid. The maximum energy available in the sodium gradient in this case is: $E_{Na} = RT \ln[(Na^+)_e/(Na^+)_i] - FV$, where $(Na^+)_e$ is the extracellular concentration of sodium, $(Na^+)_i$ is the intracellular concentration of sodium, F is the Faraday constant (23 cal/mole-mV) and V is the membrane potential in mV (outside taken as zero potential). The variables are either known or can be estimated for *M. californianus* gill tissue. $(Na^+)_e$ is 425 mEq/liter in MBL (Cavanaugh, 1956). Potts (1958) reported that the sodium concentration in the sarcoplasm of several muscle types from *M. edulis* was approximately 80 mEq/liter. Murakami and Takahashi (1975) found a more or less steady resting potential of about 60 mV (inside negative) in the lateral cells from isolated gills of *M. edulis*. Using these figures, the potential energy available from the electrochemical gradient of sodium is approximately 2340 cal/mole. The energy required to maintain the amino acid gradient is: $E_{aa} = RT \ln[(aa)_i/(aa)_e]$, where $(aa)_i$ is the intracellular concentration of amino acid, and $(aa)_e$ is the extracellular concentration at steady state. Provided that coupling of the flows of sodium and amino acid were 100% efficient, the potential energy from the sodium gradient could account for the maintenance of a chemical gradient of amino acid of approximately 60:1.

This calculation is based on what may be overgenerous assumptions. Geck and Heinz (1976) argue that although an electrical potential difference is thermodynamically equivalent to a chemical concentration difference, in many cases it is less effective in supplying energy for sodium-coupled transport of organic solutes. In any case, the energy supplied by the electrogenic sodium gradient is inadequate to account for an observed steady state concentration gradient of cycloleucine of 2000:1 (Fig. 4).

Thus these data both provide additional support for the presence of a sodium-coupled mechanism for transport of amino acids in *Mytilus* gill and cast doubt on the adequacy of such a mechanism to provide a full explanation for this transepidermal transport.

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SUMMARY

1. Intact mussels and an *in vitro* preparation of isolated gill tissue were employed to study characteristics of the influx and net flux of amino acids in *Mytilus californianus.*

2. The kinetics of influx of ^{14}C-labelled amino acids were complicated by the presence of a rapidly labelled extracellular compartment.

3. Correction of influx data for the extracellular compartment revealed influx of ^{14}C-cycloleucine and ^{14}C-glycine to be mediated by a transport mechanism adequately described by Michaelis-Menten kinetics. Passive diffusion plays no significant role in influx.

4. Influx and net flux of glycine into intact *Mytilus* were examined. From high concentrations (> 20 μM) the influx of ^{14}C-glycine was equivalent to the net influx of primary amine as determined by fluorescamine. At low ambient concentrations (< 20 μM), influx of ^{14}C-glycine occurred more rapidly than net influx as determined by fluorescamine. The data suggest that influx of labelled substrate is accompanied by efflux of unknown primary amine(s). In the absence of labelled substrate, efflux continues until a steady-state concentration of 2–5 μM in the medium is achieved.

5. The rate of influx of ^{14}C-cycloleucine into isolated gill tissue, and the concentration gradient which can be developed by gill tissue are reduced when sodium is replaced in the medium.

6. The efflux of primary amines from isolated gill tissue is stimulated by the replacement of sodium in the medium with choline.

7. The data are consistent with a sodium-coupled mechanism for the transport of amino acid into gill tissue. However, energetic considerations cast doubt on the adequacy of such a mechanism to account fully for the observed trans-epidermal transport.

LITERATURE CITED

ANDERSON, J. W., AND W. B. BEDFORD, 1973. The physiological response of the estuarine clam, *Rangia cuneata* (Gray), to salinity. II. Uptake of glycine. *Biol. Bull.*, **144**: 229–247.

BAMFORD, D. R., AND E. CAMPBELL, 1976. The effect of environmental factors on the absorption of L-phenylalanine by the gill of *Mytilus edulis*. *Comp. Biochem. Physiol.*, **53A**: 295–299.

BAMFORD, D. R., AND R. MCCREA, 1975. Active absorption of neutral and basic amino acids by the gill of the common cockle, *Cerastoderma edule*. *Comp. Biochem. Physiol.*, **50A**: 811–817.

CAVANAUGH, G. M., (Ed.), 1956. *Formulae and methods, IV, of the Marine Biological Laboratory chemical room.* Marine Biological Laboratory, Woods Hole, Massachusetts, 61 pp.

GECK, P., AND E. HEINZ, 1976. Coupling in secondary transport: effect of electrical potentials on the kinetics of ion linked co-transport. *Biochim. Biophys. Acta*, **443**: 49–53.

JACQUEZ, J. A., AND J. A. SCHAFER, 1969. Na^+ and K^+ electrochemical potential gradients and the transport of α-aminoisobutyric acid in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta*, **193**: 368–383.

JOHANNES, R. E., S. J. COWARD, AND K. L. WEBB, 1969. Are dissolved amino acids an energy source for marine invertebrates? *Comp. Biochem. Physiol.*, **29**: 283–288.

MURAKAMI, A., AND K. TAKAHASHI, 1975. Correlation of electrical and mechanical responses in nervous control of cilia. *Nature*, **257**: 48–49.

NORTH, B. B., 1975. Primary amines in California coastal waters: Utilization by phytoplankton. *Limnol. Oceanogr.*, **20**: 20–27.

PÉQUIGNAT, E., 1973. A kinetic and autoradiographic study of the direct assimilation of amino acids and glucose by the organs of the mussel *Mytilus edulis*. *Mar. Biol.*, **19**: 227–244.

POTTS, W. T. W., 1958. The inorganic and amino acid composition of some lamellibranch muscles. *J. Exp. Biol.*, **35**: 749–764.

SCHULTZ, S. G., AND P. F. CURRAN, 1970. Coupled transport of sodium and organic solutes. *Physiol. Rev.*, **50**: 637–718.

STEPHENS, G. C., 1972. Amino acid accumulation and assimilation in marine organisms. Pages 155–184 in J. W. Campbell and L. Goldstein, Eds., *Nitrogen metabolism and the environment*. Academic Press, New York.

STEPHENS, G. C., 1975. Uptake of naturally occurring primary amines by marine annelids. *Biol. Bull.*, **149**: 397–407.

STEPHENS, G. C., AND R. A. SCHINSKE, 1961. Uptake of amino acids by marine invertebrates. *Limnol. Oceanogr.*, **6**: 175–181.

STEWART, M. G., AND D. R. BAMFORD, 1975. Kinetics of alanine uptake by the gills of the soft shelled clam, *Mya arenaria*. *Comp. Biochem. Physiol.*, **52A**: 67–74.

UDENFRIEND, S., S. STEIN, P. BÖHLEN, W. DAIRMAN, W. LEIMGRUBER, AND M. WEIGLE, 1972. Fluorescamine: a reagent for assay of amino acids, peptides, and primary amines in the picomole range. *Science*, **178**: 871–872.

WRIGHT, S. H., 1976. Isolated gills as a system for the study of amino acid uptake by *Mytilus*. *Am. Zool.*, **16**: 237.

WRIGHT, S. H., T. L. JOHNSON, AND J. H. CROWE, 1975. Transport of amino acids by isolated gills of the mussel *Mytilus californianus* Conrad. *J. Exp. Biol.*, **62**: 313–325.

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CONTENTS

BRADSHAW, WILLIAM E. AND CHRISTINA M. HOLZAPFEL
Interaction between photoperiod, temperature, and chilling in dormant larvae of the tree-hole mosquito, *Toxorhynchites rutilus* Coq.. 147

CONKLIN, EDWIN J., CHARLES H. BIGGER AND RICHARD N. MARISCAL
The formation and taxonomic status of the microbasic q-mastigophore nematocyst of sea anemones.......................... 159

ELY, MELVIN J., JR. AND ARTHUR M. JUNGREIS
Radiation-induced inhibition of eclosion in the tobacco hornworm, *Manduca sexta*.................................... 169

MCMAHON, ROBERT F. AND W. D. RUSSELL-HUNTER
Temperature relations of aerial and aquatic respiration in six littoral snails in relation to their vertical zonation...................... 182

PAYEN, GENEVIEVE G. AND JOHN D. COSTLOW
Effects of a juvenile hormone mimic on male and female gametogenesis of the mud-crab, *Rhithropanopeus harrisii* (Gould) (Brachyura : Xanthidae)................................ 199

POTSWALD, HERBERT E.
Further observations on the structure and function of the operculum in *Spirorbis moerchi* (Serpulidae : Spirorbinae)................ 209

RAM, JEFFREY L.
Hormonal control of reproduction in *Busycon* : laying of egg capsules caused by nervous system extracts............................ 221

SCHWAB, WALTER E.
The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita*. I. Electrophysiological analysis........................ 233

SCHWAB, WALTER E.
The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita*. II. The effects of ions and drugs...................... 251

WEIS, JUDITH S.
Limb regeneration in fiddler crabs: species differences and effects of methylmercury.................................... 263

WHITLATCH, ROBERT B.
Seasonal changes in the community structure of the macrobenthos inhabiting the intertidal sand and mud flats of Barnstable Harbor, Massachusetts.................................... 275

WRIGHT, STEPHEN H. AND GROVER C. STEPHENS
Characteristics of influx and net flux of amino acids in *Mytilus californianus*.................................... 295

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THE

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THE BIOLOGICAL BULLETIN accepts original research reports of intermediate length on a variety of subjects of biological interest. In general, these papers are either of particular interest to workers at the Marine Biological Laboratory, or of outstanding general significance to a large number of biologists throughout the world. Normally, review papers (except those written at the specific invitation of the Editorial Board), very short papers (less than five printed pages), preliminary notes, and papers which describe only a new technique or method without presenting substantial quantities of data resulting from the use of the new method cannot be accepted for publication. A paper will usually appear within four months of the date of its acceptance.

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1. *Manuscripts.* Manuscripts must be typed in double spacing (*including* figure legends, foot-notes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. They should be carefully proof-read before being submitted and all typographical errors corrected legibly in black ink. Pages should be numbered. A left-hand margin of at least 1½ inches should be allowed.

2. *Tables, Foot-Notes, Figure Legends, etc.* Tables should be typed on separate sheets and placed in correct sequence in the text. Because of the high cost of setting such material in type, authors are earnestly requested to limit tabular material as much as possible. Similarly, foot-notes to tables should be avoided wherever possible. If they are essential, they should be indicated by asterisks, daggers, etc., rather than by numbers. Foot-notes are not normally permitted in the body of the text. Such material should be incorporated into the text where appropriate. Explanations of figures should be typed double-spaced and placed on separate sheets at the end of the paper.

3. *A condensed title* or running head of no more than 35 letters and spaces should be included.

Continued on Cover Three

Vol. 152, No. 3 June, 1977

THE

BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

GEOGRAPHIC ORIENTATION, TIME AND MUDSNAIL PHOTOTAXIS

Reference: *Biol. Bull.*, **152**: 311–324. (June, 1977)

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A series of investigations of mudsnails in our laboratories led to the conclusion that these snails were remarkably sensitive to the atmospheric electromagnetic fields. They exhibited orientational modifications in response to experimentally altered directions and strengths of very weak magnetic fields (Brown, Brett, Bennett, and Barnwell, 1960; Brown, Bennett and Webb, 1960; Brown, Webb, and Brett, 1960; Barnwell and Brown, 1961; Brown, Webb, and Barnwell, 1964). Indeed, there appeared to be solar day, lunar day, and semimonthly variations in their responsiveness to specific parameters of such fields. Not only was their responding mechanism geared to deal most effectively with fields very close to natural ambient strengths, but the snails were able to adjust or accommodate in a short time and then respond well to an experimentally altered strength (Brown, Barnwell, and Webb, 1964). Systematic variation in magnitudes of responses to horizontal magnetic fields with vector directions parallel and at right angles to their bodily axis in a fixed geographic orientation led to a hypothesis that the responding system was acting as a directional dual antenna (Brown, 1960). The snails were behaving as if one component was rotating within the body in a manner correlated with the earth's rotation relative to the sun, and the other component, relative to the moon.

Other experiments strongly suggested that the snails could also perceive and distinguish directional vectors, separately, of the electrostatic field (Brown, Webb and Schroeder, 1961) and high energy radiation (Brown, and Webb, 1968) of their environment. Comparable studies with the flatworm, *Dugesia*, suggested that such biological responsiveness was widespread (Brown, 1971).

The present investigation was designed in order to learn more concerning the natural behavior of the snails as it varied with time of day and month, and with four geographic orientations, and to discover any other systematic variations which

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might be present. It seemed probable that further information concerning biological clocks and geographic orientation would be disclosed. With the remarkable similarities in clock and compass phenomena throughout the spectrum of life, any discoveries in the mudsnail would be expected to extend our knowledge of the basic nature of the phenomena in organisms in general.

MATERIALS AND METHODS

The abundant marine mudsnail, *Nassarius obsoletus*, was obtained during each of four summers from a single small tidal area in Chappoquoit Marsh, West Falmouth, Massachusetts. About 300 animals were collected twice each week, Mondays and Fridays, with each collection kept separate on tables in running sea water. During the first year (1972), the experiments were performed in a second-floor room with southerly exposure in the Lillie Building at the Marine Biological Laboratory. The stocks were maintained on a lead-lined sea table. The remaining studies (1974, 1975 and 1976) were conducted in two different but similar laboratories with southerly exposure, on the second floor of Whitman Building, with the collections maintained on fiberglass sea tables. The rooms were air-conditioned to essentially constant temperature. In 1976, for example, the early AM temperatures averaged 20.6 ± 0.7° C; and the PM ones, 21.4 ± 0.8° C. The snails collected on Mondays were observed in experiments the following Thursdays and Fridays; those collected on Fridays were observed the following Mondays, Tuesdays, and Wednesdays. After use each collection of snails was discarded.

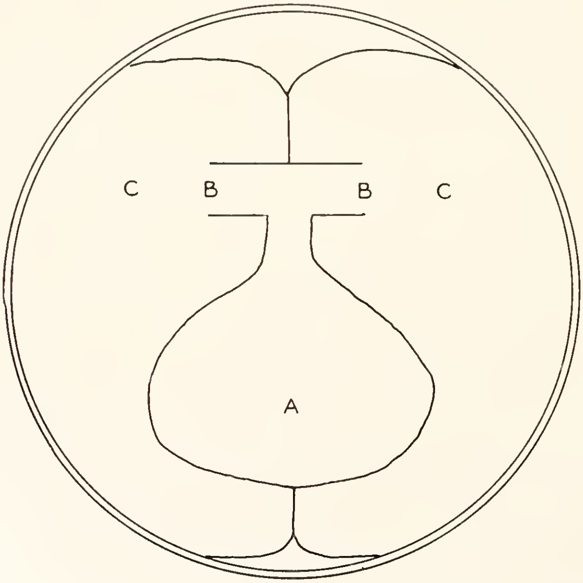

FIGURE 1. View from above one of four identically constructed orientation chambers for the snails—a 23 cm cylindrical glass crystallizing dish with an aluminum frame inserted. The snails initially placed in A make their way out through B into C.

The experiments comprised picking at random forty snails from the stock supply and placing ten in each of four special containers, constructed to be as closely identical as possible. These containers were cylindrical glass crystallizing dishes 23 cm in diameter with vertical 8 cm sides, containing aluminum inserts made from 40 × 10 cm sheets folded in half lengthwise, the two halves freed from one another by a cut close to the fold except over two short sections, and then given the form noted in Figure 1. Continuity at B in the figure was accomplished by leaving the upper half of each side uncut to produce an escape passage about 2.5 cm in height. These inserts were held firmly in place by the spring of the metal. The neck of the center corral, A, was kept with constant width by a small aluminum saddle.

One such container was placed in each of four plywood boxes with light-tight but easily removable covers. The exits for the starting points (A in Fig. 1) for the snails were directed in each of the four cardinal compass directions. When covered, the snails received illumination only from their right or their left from a 25-Watt frosted lamp by way of 3 cm circular apertures in the box at right angles to the path of the snails as they were ready to exit. Exiting snails went in large majority toward the light, whichever side it chanced to be, indicating a persistent, moderately strong, positive phototaxis.

Each morning, five days a week, between about 7:30 and 9:30, and again each afternoon between about 12:30 and 14:30 Eastern Standard Time, every dish of ten snails was exposed for a carefully measured ten minutes apiece to each of eight conditions: pointed in each of the four directions with the light on the right side and with the light on the left side. The number of snails that had emerged into C of Figure 1, toward the light and away from it, for each light direction was recorded. The containers with their snails were then moved and observed again in different geographic directions in opposite rotational direction between one day and the next. The order of offering the lights, right and left, also alternated between days.

The data for each half day thus contained as the mean for the forty-snail sample, the differing degrees of tendency to move, the differing tendencies of snails to emerge among the four compass directions, and the differing strengths of the phototactic response. The snails, when moving (rate ranged from 0.14 to

TABLE I

Diurnal differences in number of snails active.

Year	Mean number emerging		PM as % of AM	Number of days		Number of days
	AM	PM		AM > PM	PM > AM	
1972	83.8	70.8	84.5	26	18	44
1974	208.1	167.4	80.4	42	8	50
1975	163.5	134.1	82.0	45	13	58
1976	179.1	150.9	84.3	39	11	50
Mean	158.6	130.8	82.8			
Sum				152	50	202

0.23 cm/sec) could easily reach C of Figure 1, and the shape of the corral prevented snails from re-entering B during the ten minutes.

Any unintentional bias introduced by differences between specific boxes or specific lights was minimized by complete shuffling of the boxes among directions and also the light bulbs of the boxes between one year and the next.

The experiments were conducted on 44 days in 1972 (June 26 through August 24), 50 days in 1974 (June 17 through August 23), 58 days in 1975 (June 10 through August 28) and 50 days in 1976 (June 14 through August 20). The maximum total possible number of snail exits for AM or PM, over the four years was 64,640 with 8080 different snails, or 320 per half day for 40 snails for 202 days.

RESULTS

All snails

There was a mean diurnal variation in the total number of snails emerging. For each of the four summers only about 83% as many snails emerged into C (Fig. 1) in the afternoon as in the morning (Table I). The consistency of this ratio among the four years emphasized the high statistical significance which is also evident from an examination of the number of days on which more emerged in the morning than afternoon ($\chi^2 = 51.5$, d.f. = 1, $P < 0.001$). There appeared to be no significant difference between the percentages of animals responding negatively to the light between AM and PM. The mean for afternoon for the four years was about 96% of morning. However, there were highly significant differences among the four years in the mean daily percentage of negatively phototactic snails (Table II). The latter percentages tended to be negatively correlated with the number of active snails ($r = -0.91$, $t = 3.04$, $P < 0.06$).

The day by day differences in total numbers of snails emerging between morning and afternoon for each of the three years of study in the Whitman Building (1974, 1975 and 1976) appeared to display a mean, essentially bimodal, synodic monthly variation. The minima tended to fall four to five days following both new moon and full moon. Analysis of variance of four successive segments of the month (7, 10, 6, and 7 days commencing at new moon) yielded $F = 9.19$, d.f. = 3, and $P < 0.001$. The pattern of this mean synodic monthly cycle for the data for the three years (Fig. 2) coincided remarkably with the mean synodic monthly cycle of water uptake of pinto bean seeds during their first four hours (over the noon hour) in water as found by two observers independently assaying daily the

TABLE II

Average number of snails active per day and percentages of negatively phototactic snails.

Year	Mean daily % negative	Mean number active/day
1972	18.05 ± 0.919	154.6
1974	10.62 ± 0.502	375.5
1975	12.90 ± 0.493	297.6
1976	8.40 ± 0.414	330.0

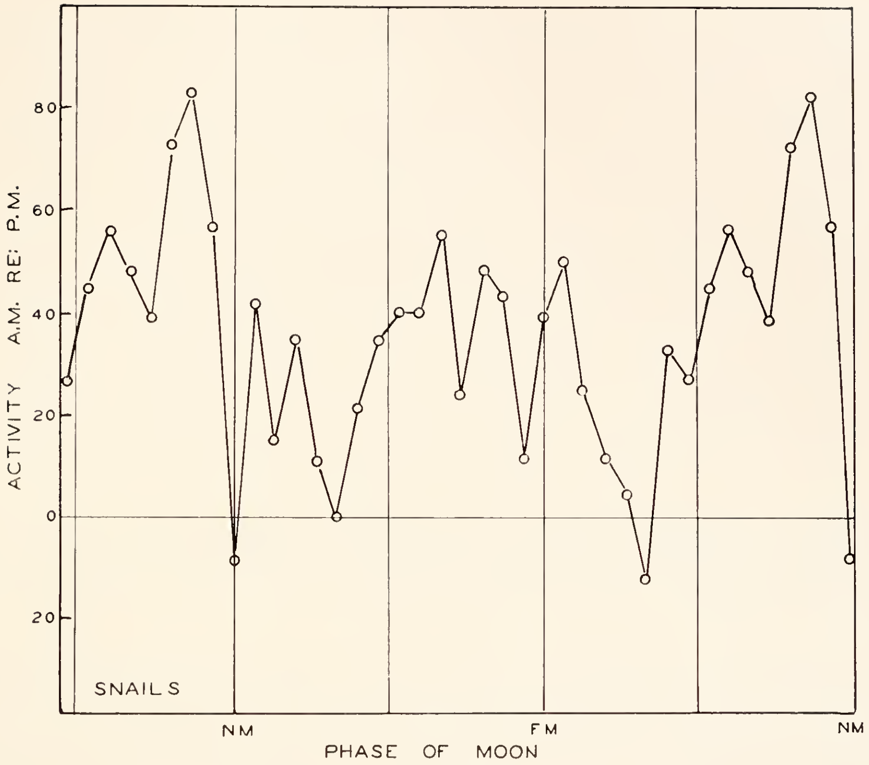

FIGURE 2. The mean differences in numbers of active snails between AM and PM for days of the synodic month for the three summers, 1974 through 1976; NM is new moon; FM, full moon.

water uptake (percentage weight increase of the beans) as averages of 32 twenty-bean samples concurrently in the same laboratory during the summer of 1976 (Fig. 3). The similarity between these snail and bean synodic monthly cycles was interpreted to render it improbable that the snails had obtained this cycle from the periodism of the tides on the beach from which they had been collected three to five days earlier and thereafter were maintaining the cycle autonomously by some endogenous oscillating system. Moreover, a comparison of the mean monthly variation of the same AM–PM difference for the snails during 1972 with that for the data of Figure 2, taking ten independent consecutive three-day means of each to compensate for the paucity in data comprising the 1972 cycle, yielded $r = -0.647$, $N = 10$, and $P < 0.05$. The 1972 monthly pattern was approximately mirror-imaging that found for the later three summers.

Negatively phototactic snails

Examination of the mean monthly patterns of variation in the percentage of negatively phototactic snails for all data for each of the two halves of the day

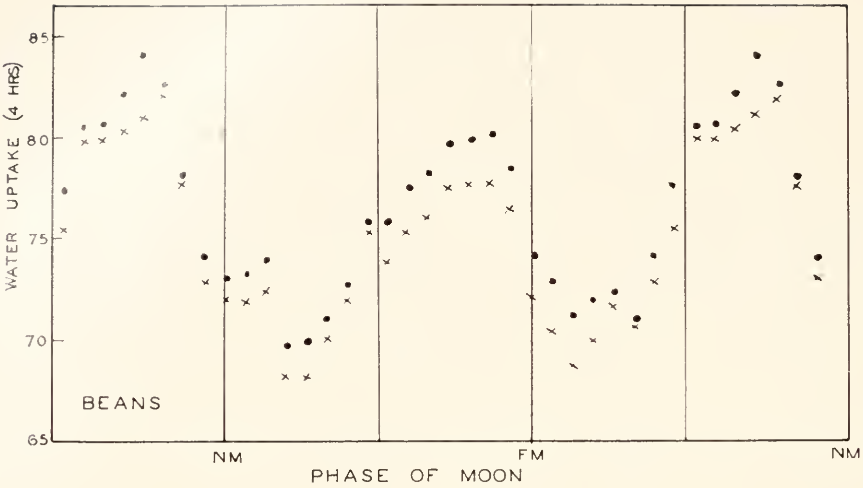

FIGURE 3. The monthly variation in 1976 in increase in weight of Pinto beans by water absorption over a carefully measured four-hour interval, as measured by two observers in the same laboratory at the same times at which the snails were being studied. These are three-day moving means; NM is new moon; FM, full moon.

disclosed these to be moderately similar to one another. The coefficient of correlation between the two independent AM and PM series was +0.636, t = 4.37, N = 30, and $P < 0.001$. The mean monthly pattern for the averaged AM and PM percentages for the four years was negatively correlated with the four-year mean monthly pattern of all emerging snails, with r = −0.51, t = 3.14, N = 30, and $P < 0.005$. An analysis of variance for the percentages of negatively phototactic snails using both AM and PM patterns for four successive segments of the month (12, 5, 8, and 5 days commencing the sixth day following full moon) yielded F = 4.77, d.f. = 3, N = 60, and $P < 0.01$. Two minima were evident and occurred in the same lunar relationships as shown in Figure 2. The ranges of three-day moving means were 38% and 57% of the averages for AM and PM, respectively.

Of great importance was the fact that no single year of data even remotely suggested positive correlation between the AM and PM patterns. The similarity gradually became evident with the increasing years of accumulated data, strongly suggesting that the similarity appeared as a common, specifically patterned, monthly variation which became apparent only with reduction of noise with the increasing amounts of pooled data.

During investigation of the numbers of negatively phototactic snails for each compass direction a highly significant difference was found among them. As seen in Figure 4, the minimum negative behavior was noted when the snails were directed east with light from north or south. A maximum in negative responses was observed for south-directed animals with illumination from the east or west. The coefficient of correlation, r, between the geographic morning and afternoon patterns for the four years (Table III) was +0.872, t = 6.66, N = 16, and $P <$ 0.001. This last high correlation also incorporated the significant differences among the years in total activity. A χ^2 test for the difference between the totals for the east and south negative responses yielded 14.7, d.f. = 1, and $P < 0.001$. For AM and PM separately, the differences gave $\chi^2 = 7.72$ and 6.99, respectively, with $P < 0.01$ for each. In the combined data for AM and PM, north and east differed from south and west, $\chi^2 = 16.36$, d.f. = 1, and $P < 0.001$. Clearly, the effect of the illumination in orienting the snails varied with the compass direction of the paths as the snails encountered the right-angle lights from their right or left sides.

Another analysis was made for all the data on snails that had responded negatively to the lights. First, comparing all the negative responses when the light was from the left, numbering 3306, with those when the light came from the right, numbering 2974, yielded a χ^2 of 17.55, d.f. = 1, and $P < 0.001$. The same difference was evident for both AM ($\chi^2 = 9.58$, $P < 0.01$) and PM ($\chi^2 = 7.98$, $P < 0.01$) separately. The negative response was, on the average, about 11.1% stronger when the light was from the left than when from the right. However, this percentage

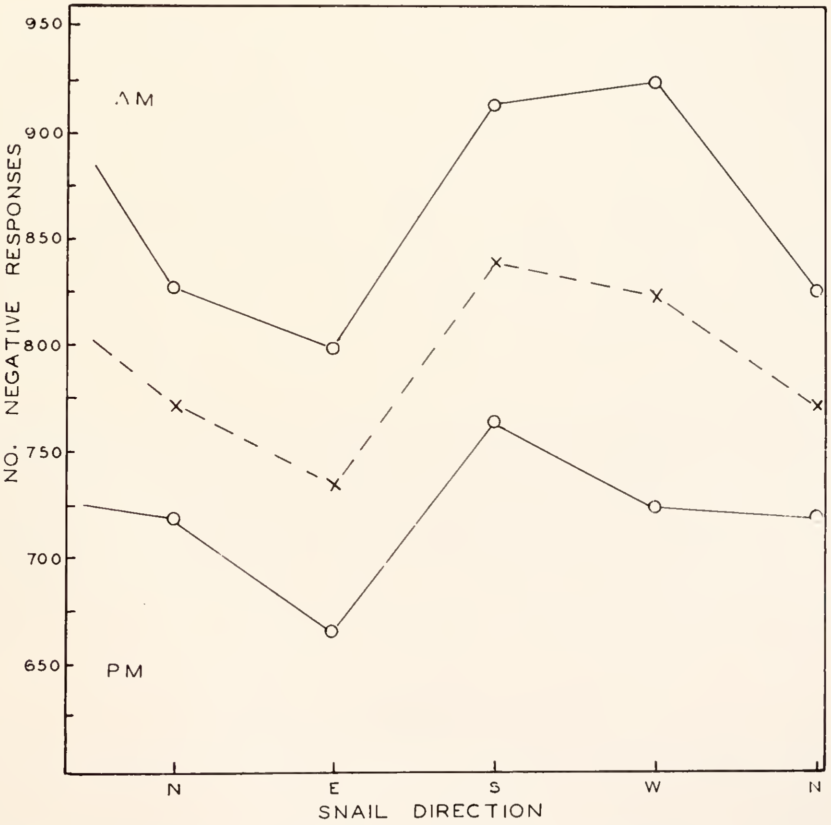

FIGURE 4. The numbers of negatively phototactic snails emerging for each geographic direction for AM and PM over the four summers of study. Abscissa indicates the direction of snail movement upon encountering a light from the right or left. The broken line describes the means for AM and PM.

TABLE III

Numbers of negatively phototactic snails.

	Year	N	E	S	W
AM	1972	137	143	195	165
	1974	212	214	245	252
	1975	306	287	315	307
	1976	171	155	159	201
				Total: 3464	
PM	1972	113	124	167	117
	1974	228	183	215	201
	1975	226	197	222	252
	1976	151	161	161	156
				Total: 2874	

varied systematically with compass direction. In the mornings this was greatest for north-directed snails (29.5%) and least for east-directed ones (−3.8%). It was 3.3% for south and 6.4% for west. Afternoons, quite differently, it was greatest for south-directed snails (19.1%) and least for north-directed ones (5.3%). The remaining directions gave intermediate values, 11.0% for east and 6.4% for west. The afternoon maximum in preference for the left over the right light had shifted about 180° relative to the morning maximum. Correlating the two half-day patterns of relative strengths of negative response with one another yielded a statistically significant correlation only with a 180° phase shift between them ($r = +0.956$, $t = 4.61$, $N = 4$, $P < 0.02$).

Positively phototactic snails

Even more striking findings from this study came from an analysis of the choices among the geographic directions. Expressing the total positively phototatic emergences for each direction as percentages of the total emerging with positive phototaxis for each half day, the values could be exprssed as deviations from 25%. This resulted in four intercorrelated values for each half day but fully independent values between AM and PM. It was evident that the variances were greater among directions in the afternoon than morning (Table IV) for each of the four years. For the pooled data the significance was great ($N = 808$, $P < 0.001$).

TABLE IV

Variances among directional choices.

Year	Var. AM	Var. PM	F_{max}	N	*P*
1972	15.704	23.422	1.491	176	<0.01
1974	13.869	19.332	1.394	200	<0.01
1975	22.629	32.884	1.453	232	<0.01
1976	17.479	20.490	1.172	200	<0.20

TABLE V

Deviations of mean paths from 25.00%.

	Year	N	E	S	W
AM	1972	−1.97	+0.22	+1.28	+0.72
	1974	−0.02	−0.52	+0.23	+0.30
	1975	−0.54	+1.30	−0.75	−0.27
	1976	−0.96	−0.05	+0.63	+0.47
		−0.87	+0.24	+0.35	+0.31
PM	1972	−2.92	+5.48	−0.33	−2.38
	1974	−0.32	+0.13	+0.63	−0.47
	1975	+0.53	−0.87	+0.03	+0.38
	1976	−0.94	+1.33	+0.48	−0.75
		−0.91	+1.52	+0.20	−0.81

A most novel discovery, however, arose from an attempt to demonstrate a correlation between the mean patterns of directional choices for mornings with afternoons. An inspection of the data for the summer of 1972 had suggested that no correlation existed between the mean morning and afternoon values direction by direction (Table V, Fig. 5A), but that a positive correlation was suggested between the directional patterns for the two times of the day when the afternoon pattern was displaced clockwise (from above) by 90° (Fig. 5B). A significant negative correlation occurred for a 270° displacement (Table VI). In viewing the data for the next three years, it was discovered that there was a positive correlation of the same general magnitude for each year between the morning patterns and the afternoon ones after a 90° clockwise displacement of the latter ($r = +0.677$, $t = 2.926$, $N = 12$, $P < 0.004$) despite no correlation between the same directions ($r = -0.155$, $t = 0.279$, $N = 12$). Because of the correlated nature of the four directional values the remaining two degrees of rotation, 180° and 270° would be expected to average a negative value. A 180° phase shift of the PM values yielded $r = +0.009$, $t = 0.028$, $N = 12$, and a 270° shift gave $r = -0.535$, $t = 2.00$, $N = 12$. When the 1972 data were included, the corresponding r-values were those listed in Table VI.

TABLE VI

Coefficients of correlation, r, between AM *and* PM *patterns.*
PM *patterns shifted clockwise relative to* AM *ones.*

Phase shift CW	1972	1974	1975	1976	All years
0°	+0.295	−0.104	−0.877	+0.340	−0.176
90°	+0.660	+0.715*	+0.646	+0.794	+0.616***
180°	+0.021	+0.357	+0.182	−0.171	+0.043
270°	−0.976**	−0.968**	+0.048	−0.963**	−0.811****

* $P < 0.05$ for pooled 1972–74; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$.

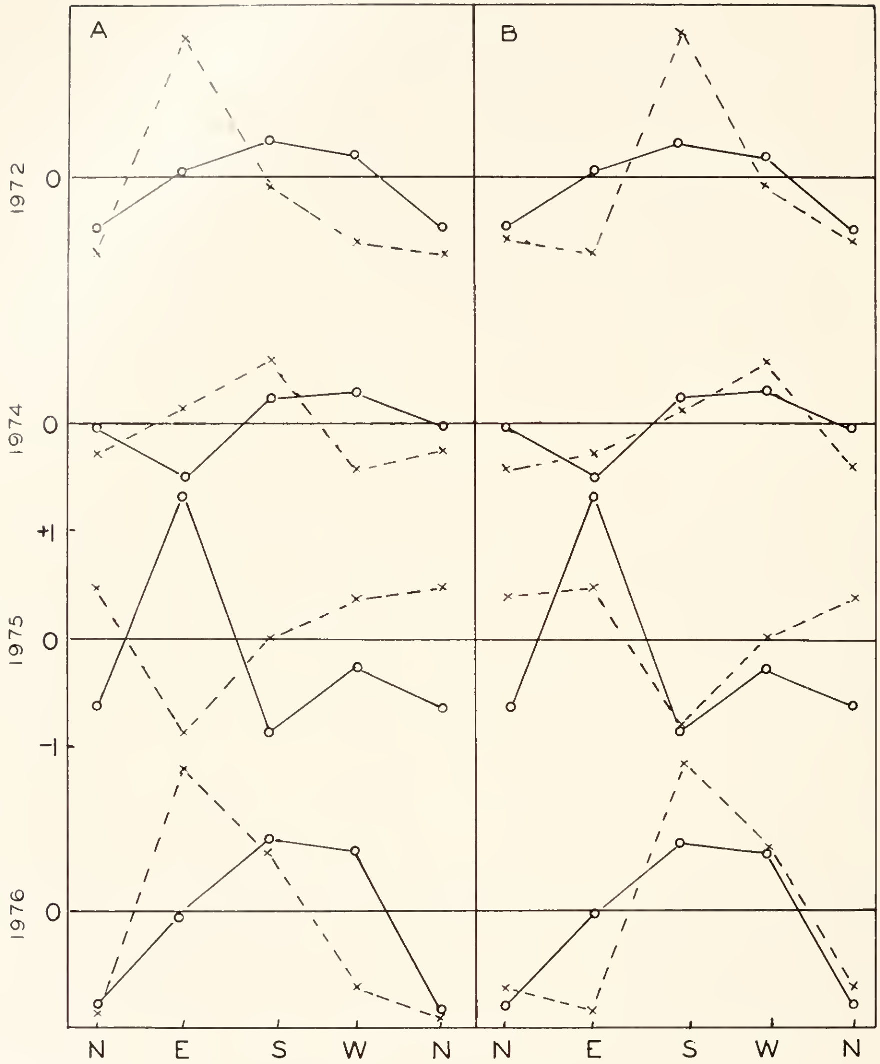

FIGURE 5. The directional preferences of snails during four years, mornings (solid line) and afternoons (broken line). The 1972 patterns are plotted at 25% of the range of the others. The ranges are shown as percentage points on the 1975 ordinate: A, all geographic directions as shown on abscissa, and B, AM values as shown on abscissa; PM values all shifted 90° clockwise (from above).

It seems significant that a maximum positive correlation and a maximum negative correlation for the four years are 180° apart (90° and 270°), and both were found to be significantly different, as means, from the noncorrelated values found for 0° and 180°. This suggests that it is not simply a directional preference but an axial preference which is shifting counterclockwise 90° between AM and PM.

Except for the year 1975 the tendency to select one geographic direction is associated with a tendency to avoid the opposite direction. It is unfortunate that the study could not have included 1973; possibly a similar anomaly to 1975 might have occurred, suggesting that the phenomenon alternated between one year and the next.

Discussion

The snails exhibited a mean 24-hour variation in essentially the same relation to time of day throughout all four summers that they were studied. This was easily explained since the snails experienced the natural day-night changes of the laboratory. This daily cycle included a relatively uniform mean reduction in activity between morning and afternoon and the difference, in turn, showed a bimodal synodic monthly pattern of modulation through 1974, 1975 and 1976. A close similarity of this pattern to that of bean water absorption occurring in the same laboratory during the summer of 1976 pointed to a subtle exogenous origin.

The mean monthly patterns of the snails through the five-year span of this study tended significantly to display a common bimodal form with minima occurring between new moon and first quarter and between full moon and third quarter. However, for some measured parameters, or at certain times, a monthly pattern for the snails was registered which was negatively correlated, with high statistical significance, with the more typical one. Minima and maxima had exchanged places. Inversion of geophysically dependent patterns including both lunar day and monthly ones has been reported between different species, within a single species at different times, and even concurrently within a single species under slightly different experimental conditions (Brown, 1960; Brown and Chow, 1973, 1976). Such inversions comprise a phenomenon which is probably commonplace. It is postulated that the inverting tendency reflects the organisms' sign and strength of response to an effective atmospheric factor which is capable of being altered, even tipped between positive and negative, with changing physiological state of the organism and by effects of other uncontrolled, or imposed, environmental conditions. Indeed the sign has been described to differ between one portion of a lunar or solar cycle and another (Brown, 1960; Brown, 1962b).

The similarity of the four-year mean monthly patterns found for the negatively phototactic snails between AM and PM, together with the absence of any comparable similarity between these patterns for the single years, bespeaks a continuing input to the snail population of a detailed exogenous, noisy, monthly pattern. This can be compared in principle with the reproducible low amplitude bimodal monthly patterns of modulation of a circadian rhythm observed for hamsters during a two-year study (Brown and Park, 1967). A common mean residual pattern, differentiating specific days in a lunar month must have persisted throughout this study. The highly significant correlation denies random monthly sequences of points.

The variation in strength of negative phototaxis with differing geographic direction of movement of the snails suggests within the animals an interaction between the oriented visible light and a polarized, subtle geophysical field within the test chambers. The latter field is presently postulated to be geomagnetism, the most regular of those known to which snails are responsive. Significant synergistic interactions between an overt vector field such as light and a vector atmospheric electromagnetic field such as geomagnetism has great implications for behavior in general.

They suggest an area for future investigations in receptor and sensory physiology and perception more broadly among organisms.

The negative responses of the snails differed between right and left lights. The response was stronger to a light on the left than on the right. This could not be due exclusively to shell coiling inasmuch as the magnitude of the difference varied systematically with the geographic direction of the snails as they encountered the lights. The pattern of this differential responsiveness with geographic direction was also 180° shifted afternoons relative to mornings. Such behavior resembles the essential inversion of the geographic directional pattern of response to a weak, right-angle, horizontal electrostatic field between morning and afternoon by the planarian, *Dugesia* (Brown, 1962a).

Among the four years the compass pattern of directional choices differed between one year and the next. But despite this, the afternoon patterns most closely resembled the morning ones each year when displaced 90° clockwise. The only manner in which these and other discovered results could be obtained is that the snails possess a system integrating the temporal daily cycle with the two-dimensional geographic compass one. Between AM and PM the patterns of directional preferences had shifted about 90° counterclockwise in the geographic compass cycle. This behavior is consistent with the earlier findings that snails could not only distinguish horizontal vector direction of magnetism but that they behaved as if a major sensor system for magnetic field rotated in a manner geared to the relative movements of sun and earth even when the sun was not visible.

The approximate quarter-cycle counterclockwise directional, or axial, preference shift between AM and PM would be an expected consequence if a sensor for magnetic vector direction relative to the longitudinal body-axis were, in effect, rotating clockwise once a day within the organism in response to an atmospheric parameter complex, reflecting the apparent clockwise motion of the sun in the northern hemisphere. To maintain a given compass orientation of its rotating sensory mechanism to the geomagnetic polarity, which especially in the absence of the sun appears to be a major orienting factor for organisms (Wiltschko and Wiltschko, 1972; Emlen, Wiltschko, Demong, Wiltschko, and Bergman, 1976), the organism must be shifting its angle of orientation relative to the geomagnetic field at a rate of 360° per day, or about 90° counterclockwise between morning and afternoon as discovered herein. It is postulated that the mechanism of clock compensation for the motion of the sun across the sky relative to the magnetic polarity of the earth is reflected here. Under the controlled conditions of this experiment, with a single fixed light with direction neutralized by equal right and left exposures, this becomes evident. Normally, with a view of the sun available for directional reference, this system would enable a consistent geographic direction to be maintained as a sun-compass response. In the absence of view of the sun and with neutralized direction of artificial lights the orientation of the organism relative to a residual stationary directional field such as geomagnetism rotates steadily counter-clockwise.

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SUMMARY

1. Mudsnails, *Nassarius obsoletus,* collected at Chappoquoit marsh, West Falmouth, Massachusetts, were observed in apparatus designed to assay samples of forty snails both mornings and afternoons for their activity, directional preference, and sign and strength of phototaxis.

2. Assays were made during two to three months over each of four summers (1972, 1974, 1975 and 1976).

3. Activity was less, and variances among choices of directions was greater, during afternoons than mornings.

4. A mean monthly pattern was described for the difference between AM and PM activities for all snails; for only negatively phototactic snails a comparable monthly pattern for AM was highly significantly correlated with one for PM. The monthly pattern of negatively phototatic snails *negatively* correlated with the monthly pattern of total snail activity.

5. The strength of negative phototaxis, as well as differential influences between right and left lights, differed with geographic oriention of the snails and indicated an integration within the snails of response to light and to a horizontally polarized subtle geophysical field.

6. For positively phototactic snails a highly significant positive correlation between the AM and PM patterns of directional choices occurred only when PM compass patterns were rotated 90° clockwise relative to AM ones, with a similarly highly significant negative correlation occurring for 270°.

7. This 90° shift is interpreted to result from orientation to geomagnetism employing a "rotating magnetic sensor" in the snails which is normally used for sun-compass compensation.

LITERATURE CITED

Barnwell, F. H., and F. A. Brown, Jr., 1961. Magnetic and photic responses in snails. *Experientia,* **17**: 513-515.

Brown, F. A., Jr., 1960. Response to pervasive geophysical factors and the biological clock problem. *Cold Spring Harbor Symp. Quant. Biol.,* **25**: 57-71.

Brown, F. A., Jr., 1962a. Response of the planarian, *Dugesia,* to very weak horizontal electrostatic fields. *Biol. Bull.,* **123**: 282-294.

Brown, F. A., Jr., 1962b. Extrinsic rhythmicality: A reference frame for biological rhythms under so-called constant conditions. *Ann. N. Y. Acad. Sci.,* **98**: 775-787.

Brown, F. A., Jr., 1971. Some orientational influences of non-visual, terrestrial electromagnetic fields. *Ann. N. Y. Acad. Sci.,* **188**: 224-241.

Brown, F. A., Jr., and C. S. Chow, 1973. Lunar-correlated variations in water uptake in bean seeds. *Biol. Bull.,* **145**: 265-278.

Brown, F. A., Jr., and C. S. Chow, 1976. Uniform daily rotation and biological rhythms and clocks in hamsters. *Physiol. Zool.,* **49**: 263-285.

Brown, F. A., Jr., and Y. H. Park, 1967. Synodic monthly modulation of the diurnal rhythm of hamsters. *Proc. Soc. Exp. Biol. Med.* **125**: 712-715.

Brown, F. A., Jr., and H. M. Webb, 1968. Some temporal and geographic relations of snail responses to very weak gamma radiation. *Physiol. Zool.,* **41**: 385-400.

BROWN, F. A., JR., F. H. BARNWELL, AND H. M. WEBB, 1964. Adaptation of the magnetoreceptive mechanism of mudsnails to geomagnetic strength. *Biol. Bull.*, **127**: 221–231.

BROWN, F. A., JR., M. F. BENNETT, AND H. M. WEBB, 1960. Magnetic compass response of an organism. *Biol. Bull.*, **119**: 65–74.

BROWN, F. A., JR., H. M. WEBB, AND F. H. BARNWELL, 1964. A compass directional phenomenon in mud-snails and its relation to magnetism. *Biol. Bull.*, **127**: 206–220.

BROWN, F. A., JR., H. M. WEBB, AND W. J. BRETT, 1960. Magnetic response of an organism and its lunar relationships. *Biol. Bull.*, **118**: 382–392.

BROWN, F. A., JR., H. M. WEBB, AND T. E. SCHROEDER, 1961. Organismic responses to differences in weak horizontal electrostatic fields. *Biol. Bull.*, **121**: 413.

BROWN, F. A., JR., W. J. BRETT, M. F. BENNETT, AND F. H. BARNWELL, 1960. Magnetic response of an organism and its solar relationships. *Biol. Bull.*, **118**: 367–381.

EMLEN, S. T., W. WILTSCHKO, N. J. DEMONG, R. WILTSCHKO, AND S. BERGMAN, 1976. Magnetic direction finding: Evidence for its use in migratory Indigo Buntings. *Science*, **193**: 505–507.

WILTSCHKO, W., AND R. WILTSCHKO, 1972. Magnetic compass of European robins. *Science*, **176**: 62–64.

Reference: *Biol. Bull.*, **152**: 325–336. (June, 1977)

ULTRASTRUCTURAL EVIDENCE THAT GASTROPODS SWALLOW SHELL RASPED DURING HOLE BORING

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Several investigators have demonstrated by light microscopy that shell scraped from the borehole during penetration of valves of prey by three species of muricid and naticid gastropods is swallowed and eliminated in the feces. Graham (1941) discovered that the stomach of specimens of *Nucella lapillus* which were boring the valves of *Mytilus edulis* contained minute curved flakes of crystalline material which dissolved with effervescence on the addition of hydrochloric acid, whereas these were absent from the stomach of snails not boring (see also Fretter and Graham, 1962). Graham (1953) noted further that the calcareous material in the stomach was embedded in a thick mucous material probably provided by the salivary glands and possibly also by the accessory salivary glands. Ziegelmeier (1954) was able to see white shell material passing down the esophagus of actively boring *Natica nitida,* and during subsequent defecation observed white pellets composed mainly of shell fragments in the feces. Carriker and Van Zandt (1972b), employing a contact microhydrophone to identify rasping periods, removed *Urosalpinx cinerea* from the valves of *Mya arenaria* at the end of the rasping period and found white shell raspings bound by a mucus-like substance in string-shaped pellets in the stomach. Pellets were birefringent under polarized light, became red when stained with alizarin sodium monosulfonate, and consisted of distinct solid particles amid soft mushy shell material. Fecal strings collected in dishes from snails which were boring small *Crassostrea virginica* also contained conspicuous white shell raspings which were likewise identified as calcium carbonate. Shell fragments appeared to be dissolved only slightly, but this could not be determined with certainty with the light microscope.

The purpose of this paper is to report observations on the ultrastructure of shell material rasped by *Urosalpinx cinerea follyensis* Baker from boreholes in the valves of *Mytilus edulis* Linné and transported normally to the stomach through the buccal cavity and esophagus.

MATERIALS AND METHODS

Snails used for the study came from Wachapreague, Virigina. They were maintained in the laboratory in running sea water (approximately 30–32‰, at room temperature) and bored and fed actively on the bivalves *Mytilus edulis* and *Crassostrea virginica* for several weeks prior to use in these observatons.

Duration of the period of chemical activity by the accessory boring organ and of rasping by the radula were determined in a valve model (Carriker and Van Zandt, 1972b) as follows. After a snail had bored about half way through the valve of a live *Mytilus edulis,* the free valve and flesh of the mussel were gently removed under water and the remaining half-shell boring-snail preparation was positioned, inner surface of the valve facing up and the snail suspended underneath, on a platform in slowly running sea water under a binocular microscope. The inner nacreous layer of the shell of *M. edulis* is translucent, and activity of the proboscis, radula, and accessory boring organ were visible as excavation of the borehole by the snail neared the inner surface of the valve. It was thus possible to determine accurately the duration of chemical and rasping periods.

After several preliminary anatomical dissections to locate shell raspings accurately in the stomach, three snails which were boring the valves of live *Mytilus edulis* were selected, and the snail-valve models were prepared. The mussels were 6 cm long.

The height in millimeters of each snail, duration in minutes of the period of chemical activity by the accessory boring organ prior to the rasping period, and the number of rasps during the rasping period just before the snail was removed for dissection were as follows: specimen 1, 35 mm, 55 min, 12 rasps; specimen 2, 31 mm, 11 min, 25 rasps; and specimen 3, 35 mm, 21 min, 40 rasps. Diameter of boreholes emerging on the interior surface of the valves was about 0.8 to 1 mm, and that on the exterior of the valves was approximately 1.2 mm.

As soon as the first snail was removed from the snail-valve preparation, its shell was immediately cracked, the soft parts removed and pinned on a small dissecting pan under sea water; the stomach was opened with fine dissecting instruments and the pellet of shell raspings was removed with a fine bulb pipette. The stomach of muricid gastropods is a simple sac (Fretter and Graham, 1962), so it was easy to locate the pellet precisely. About 5 min elapsed from the time the snail was removed from the snail-valve preparation and the time the pellet was taken from the stomach.

The pellet was spread evenly over the surface of a polished brass scanning electron microscope (SEM) stub and dried at room temperature. The following day, the specimen was rinsed briefly with distilled water to dissolve salt left by sea water, quickly dried again in air, and coated in vacuum with platinum-palladium. Examination of the specimen in the scanning electron microscope (JEOLCO JSM–U3) showed that the material was charging badly, so it was dried further in an oven at 105° C for six days. Because the mucoid coating on the exterior of the pellet obscured the contents, the mass was then opened with a fine needle point, and was shadowed again with platinum-palladium. There was still some charging, so a final layer of gold was applied in vacuum and this eliminated most of the charging. Pellets from the other snails were treated in the same way.

Ultrastructure of the normal valves of *Mytilus edulis* was examined as follows for comparison with the shell fragments swallowed by *Urosalpinx cinerea*. The valves of five *M. edulis*, 5 to 6 cm long, were removed from the soft parts, cleaned in water, dried, and fractured into small pieces with a hammer on a hard surface. Under a binocular microscope pieces were selected from the central area of the valves whose fractured surfaces exposed cross, side, and oblique sections of prisms from the prismatic region, and cross and oblique sections of lamellae from the nacreous region. Some of the fractured pieces were mounted on SEM stubs, coated with gold, and examined directly in a scanning electron microscope. Others were treated chemically as follows to reveal the outlines of shell units more clearly than was possible without the treatment: (a) immersion in 80% (by volume) ethylenediamine for about 20 hr at room temperature and washed with water, (see Figs. 8, 9, 11); and (b) immersion in 5.25% sodium hypochlorite (commercial

Clorox) for 2.75 hr with intermittent sonication for a total of 65 min, followed by retention and washing with water on a Millipore filter (0.45 μm pore size) with 5 to 10 lb. pressure from the vacuum pump (see Fig. 12). Specimens (a) and (b) were dried, mounted on SEM stubs, the latter still on a piece of Millipore filter, coated with gold, and examined in a scanning electron microscope. Sodium hypochlorite (Mutvei, 1970, 1972) and ethylenediamine (Zapanta and Trautz, 1961) selectively dissolved the organic matrix of the shell pieces, but did not noticeably alter the appearance of the mineral crystals.

Results

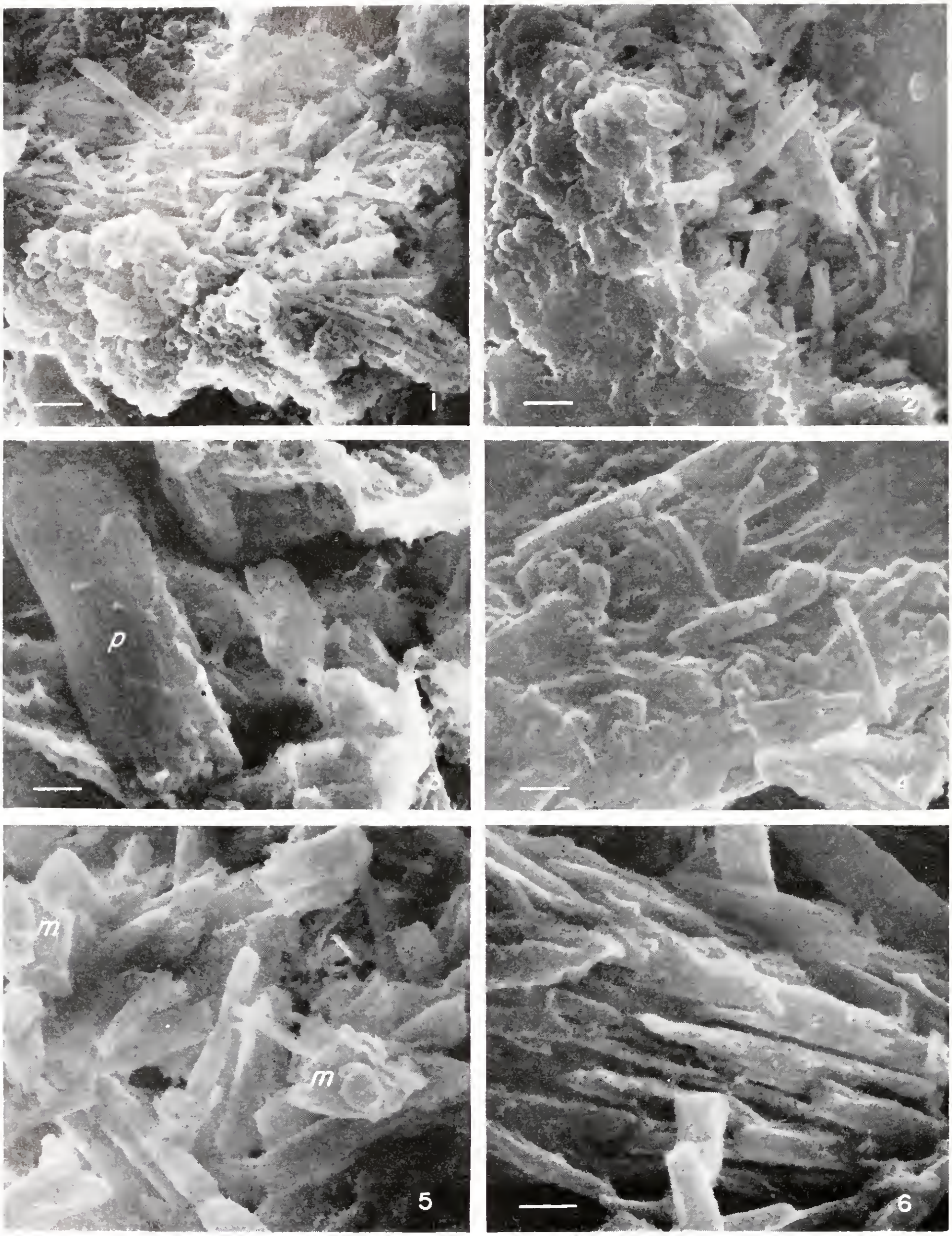
Scanning electron microscopy clearly confirmed the presence of shell fragments in the stomach of *Urosalpinx cinerea.* When large amounts of shell were removed during a rasping period, shell fragments tended to be clustered within an envelope of mucoid material and were not visible until the pellet was fractured open (Figs. 1, 2). When less shell material was scraped off by the radula, fragments tended to be embedded in the mucoid material and were more difficult to see even after the pellet was fractured (Fig. 4).

Two kinds of mineral shell units, prisms and lamellae, (Zottoli and Carriker, 1974) characteristic of the shell of *Mytilus edulis* were identified in the scanning electron micrographs. Prisms, the long slender pencil-shaped calcitic structures (Figs. 1–6) were abundant; whereas groups of the thin wafer-like aragonitic lamellae (also called tablets by some investigators) were seen only occasionally (Fig. 5).

Length of prisms in the pellets varied from pieces 15 to 18 μm (Figs. 1, 6) to small fragments (Figs. 4, 5). Bundles of prisms, still bound together by organic matrix, were also seen occasionally (Fig. 3).

As a basis for comparison of the size of prismatic shell fragments in the stomach of *Urosalpinx cinerea* with the size of prisms in undisturbed shells, the cross sectional dimensions (height and width) of 66 prisms in fractures of shell from three *Mytilus edulis* approximately 5 to 6 cm long (Fig. 13) and magnified 6000 to 10,000 times in scaning electron micrographs were measured. Sections of shell were selected in which fractures occurred at right angles to the long axes of the prisms to avoid angular distortion in the measurements. An oblique view—not at right angles to the long axis—of a fracture of prisms is illustrated in Figure 7. The cross sectional anvil shape of the prisms is characteristic. A low and a higher magnification of isolated single prisms are seen in Figures 8 and 11, respectively. The height of prisms (Fig. 13) varied from 0.6 to 2.0 μm and the width ranged from 1.1 to 2.8 μm; the arithmetic average height of prisms was 1.2 μm and the arithmetic average width was 2.1 μm. These dimensions compare favorably with those reported by Travis and Gonsalves (1969) for cross sections of prisms of the same species in electron microscopic thin sections. Several isolated single prisms (for example, Fig. 8) ranged maximally from 56 to 65 μm in length, but it is difficult to say whether this was the actual length of the prisms, or whether they were broken in the process of isolation.

The maximum "width" of the 212 prismatic fragments measured in the stomach of *Urosalpinx cinerea* (Figs. 1, 2, 4, and other micrographs not included here) ranged from 0.25 to 2.4 μm (Fig. 14). These sizes fall within the height-



FIGURES 1–6. Scanning electron micrographs of pellets of shell raspings and mucoid material removed from the stomach of *Urosalpinx cinerea* immediately after the rasping period. FIGURE 1: half a pellet opened to show interior filled with fragments of shell prisms. The dried nodular mucoid coat is on the exterior to the left; specimen 1; scale bar squals 5 μm. Figure 2: a second view of the same pellet emhasizing the mucoid coat and outlines of shell prisms; specimen 1; scale bar equals 5 μm. Figure 3: an intact bundle (p) of many shell

width dimensions of prisms measured in unrasped shell (Fig. 13), allowing some reduction in size of prisms from dissolution by the secretion of the accessory boring organ. The tapering ends and spaces among prisms in Figure 6 demonstrate the extent of dissolution that took place in the borehole prior to rasping and probably to a slight extent during passage of prisms surrounded by secretion down the esophagus to the stomach. Figures 1–5 suggest the extent of cross-sectional breakage of prisms that occurred during the rasping period (compare with Fig. 8). The "width" could represent either the height or the width of prisms, depending on their orientation in the specimens examined.

Noticeable dissolution of the organic matrix, and to some extent of the mineral portion, of prisms was evident (Fig. 6). This resulted in tapering ends and conspicuous spaces among the prisms, in marked contrast to the bundles of prisms which were uprooted before they were affected by the secretion (Fig. 3).

As a basis for comparison of the thickness of lamellar shell fragments in the stomach of *Urosalpinx cinerea* with the thickness of lamellae in undisturbed shell, the thickness (or height) of 164 lamellae in fractures of pieces of valves from three different *Mytilus edulis* approximately 5 to 6 cm long in scanning electron micrographs magnified 8000 times were measured. Sections were selected in which fractures occurred at right angles to the long axes of the lamellae (as in Fig. 10). The polygonal form of lamellae is illustrated in Figues 9 and 12. A few isolated lamellae are visible from the side in the mid-left of Figure 12, much as they appear in the stomach pellets (Fig. 5) of *U. cinerea*. Thickness of lamellae in undisturbed shell of three different individuals of *M. edulis* ranged from 0.25 to 2.0 μm (Fig. 15). Study of additional fractures of nacreous shell disclosed that the thickness of lamellae varied widely from individual to individual mussel, and that lamellae formed at the juncture of myostracum and naceous and prismatic strata were as much as one-half to one-third thinner than elsewhere in the shell. The bimodal curve in Figure 15 is indicative of the differences observed in the size of lamellae from the center of nacreous strata (not adjacent to myostracum) in three different individuals.

Only three lamellae ranging in thickness from 0.2 to 0.3 μm were seen among the shell fragments swallowed by *Urosalpinx cinerea* in Figure 5 (compare with Fig. 15).

From information now available, it is possible to estimate the quantity of shell excavated by the radula and swallowed during hole boring. Carriker (1969) estimated that the total surface area of the bottom of a borehole 1.47 mm in diameter rasped during a rasping period ranged from 1/10 to 1/5. Depth of rasp marks varies widely with the hardness of the shell (Carriker, 1969). As a basis for calculation let us assume an average depth of each rasp mark of 6 μm in the shell of *Mytilus edulis* (see Fig. 13, in Carriker, Schaadt, and Peters, 1974); an average surface area at the bottom of the borehole of 0.785 mm^2 (based on an average

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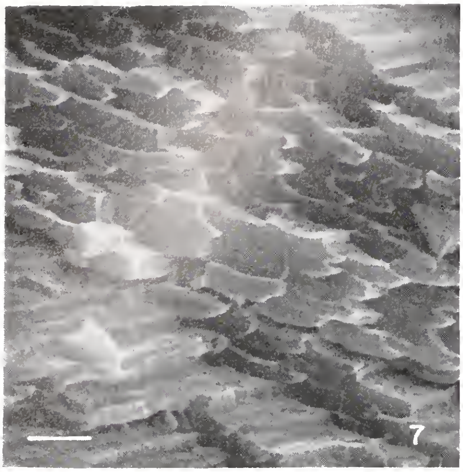
prisms dislodged by the radula from beneath the zone of dissolution in the borehole and showing no, or little, evidence of dissolution; specimen 3; scale bar equals 2 μm. Figure 4: interior of a portion of a pellet with few shell prisms embedded in mucoid material; specimen 3; scale bar equals 1 μm. Figure 5: two prominent clusters of shell lamellae (m) among a rubble of prisms; specimen 1; scale bar equals 2 μm. Figure 6: organic matrix and part of the calcareous cores of shell prisms noticeably dissolved; specimen 1; scale bar equals 1.5 μm.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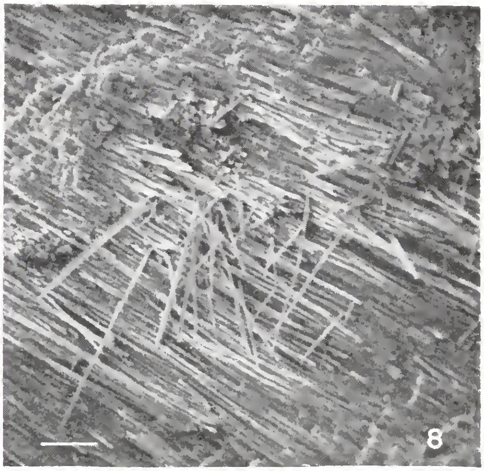
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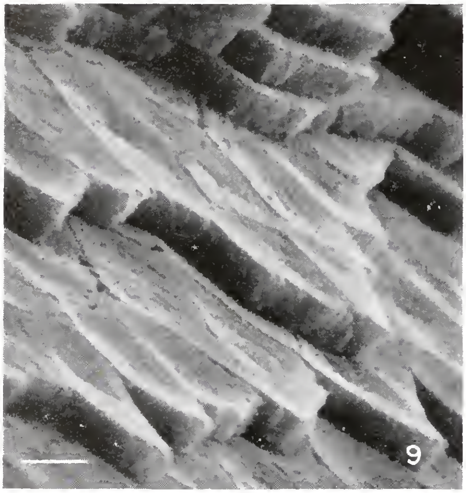
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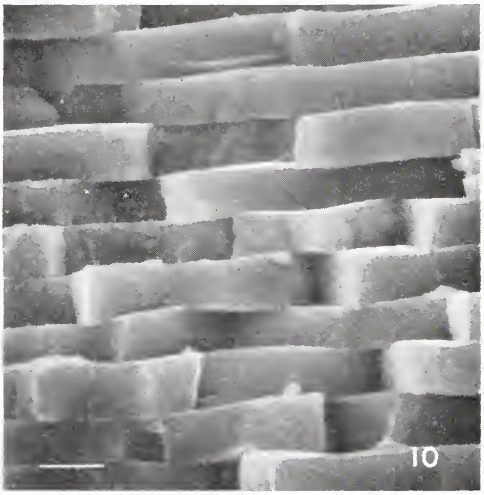
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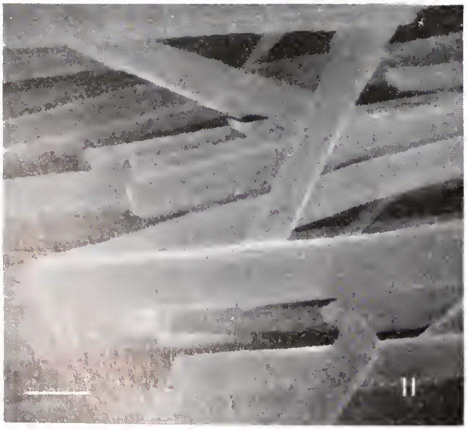
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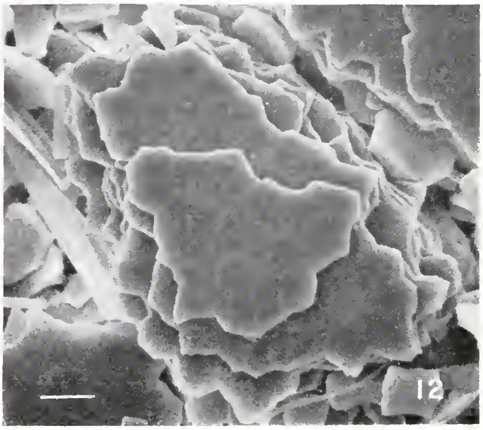
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diameter of 1 mm since the borehole tapers from exterior to interior); a total of 150 rasping periods for penetrating an oyster valve 1.0 mm thick (Carriker and Van Zandt, 1927b); and removal of shell from 15% of the surface of the borehole at each rasping period. Thus of a total of 0.785 mm^3 of shell removed from the borehole by combined chemical and mechanical activity, 0.106 mm^3 was rasped out by the radula. It follows then that swallowing accounted for roughly only 14% of the shell in the borehole. These calculations are based on gross estimates, particularly those of the amount of shell actually removed by each rasping stroke and the number of strokes per rasping period, and thus must be used with caution.

Discussion

The study provided ultrastructural evidence for the first time that *Urosalpinx cinerea* swallows shell rasped from the borehole during penetration of prey.

The predominance of prisms in the pellets indicates that snails had rasped through the outer prismatic region of the shell of *Mytilus edulis* when sacrificed for analysis of stomach contents. The scarcity of lamellae and their minute size suggest that the snails had just commenced rasping in the nacreous stratum of the shell.

The chemical composition of the mucoid envelope surrounding shell raspings in the stomach is unknown. The mucoid material may consist of a mixture of secretions from the accessory boring organ wiped from the bottom of the borehole during rasping, of mucus secreted by buccal glands, esophageal mucous glands, and possibly also of secretion from the salivary glands and the gland of Leiblein. Graham (1953) and Fretter and Graham (1962) concluded that the accessory salivary glands, as well as the accessory boring organ, seem to be necessary for hole boring as these organs are present in the shell boring muricacean stenoglossan gastropods, but are absent in the next two super-families, the Buccinacea and Volutacea. Precisely what role the accessory salivary glands play in shell excavation is unclear, but that they are involved appears a logical deduction from the position of their external aperture in the front midventral portion of the buccal cavity (Graham, 1953). At first glance absence of accessory salivary glands from predatory, shell boring, naticacean gastropods seems to negate this interpretation. However, there is a possibility that the accessory salivary glands may be functionally replaced by the collar of mucus-like secreting cells which surround naticid accessory boring organs. This provocative area of research awaits the histochemist and physiologist.

FIGURES 7–12. Scanning electron micrographs of the structure of normal shells of adult *Mytilus edulis*. Figure 7: ends and sides of prisms in a cross sectional fracture of shell, treated with phosphate buffer at pH 7; scale bar equals 2 μm. Figure 8: prismatic shell treated with ethylenediamine to free the surface prisms from the organic matrix; scale bar equals 20 μm. Figure 9: oblique view of terraces of nacre treated with sodium hypochlorite to expose boundaries of individual lamellae; scale bar equals 2 μm. Figure 10: fracture of nacre exposing cross sections (heights) of lamellae, treated with sodium hypochlorite; scale bar equals 2 μm. Figure 11: prisms freed from the organic matrix by immersion in ethylenediamine; scale bar equals 2 μm. Figure 12: clusters and single lamellae freed from nacre by treatment with sodium hypochlorite and sonication; scale bar equals 5 μm.

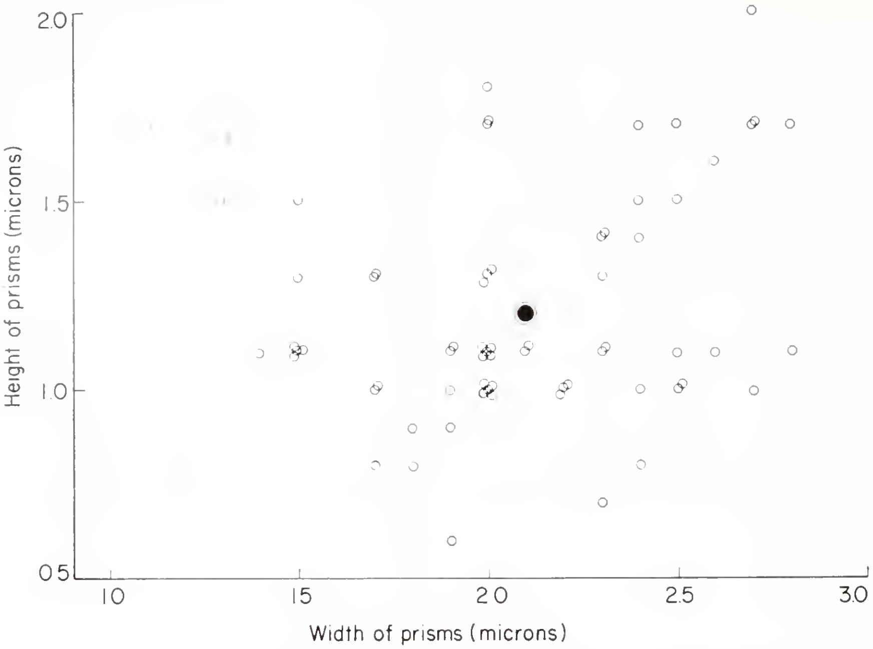

FIGURE 13. Plot of cross sectional dimensions of prisms in fractured shell of adult *Mytilus edulis;* solid circle represents arithmetic average height and width of prisms.

Most of the dissolution of shell probably takes place on the surface of the borehole prior to the rasping period (Carriker, 1969). The characteristic initial dissolution of cementing organic matrix of shell units (Carriker, 1969; Carriker,

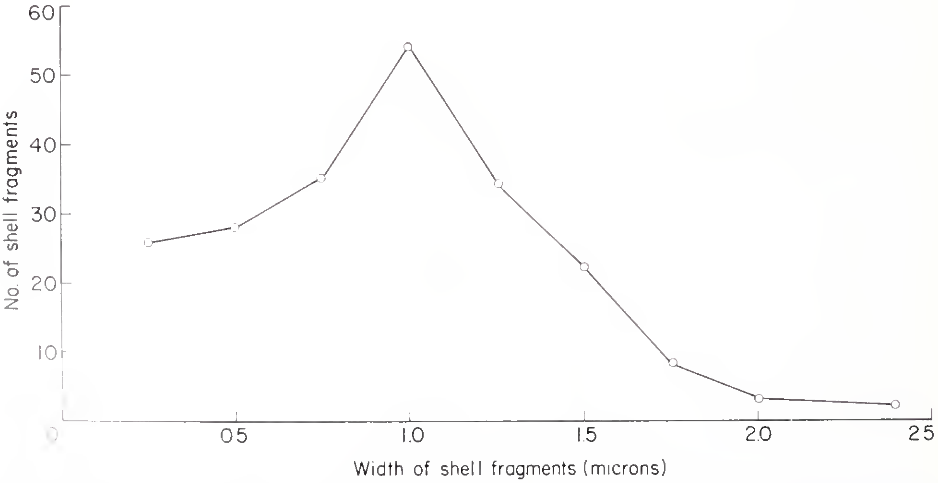

FIGURE 14. Plot of "width" of prismatic fragments taken from stomach of *Urosalpinx cinerea.*

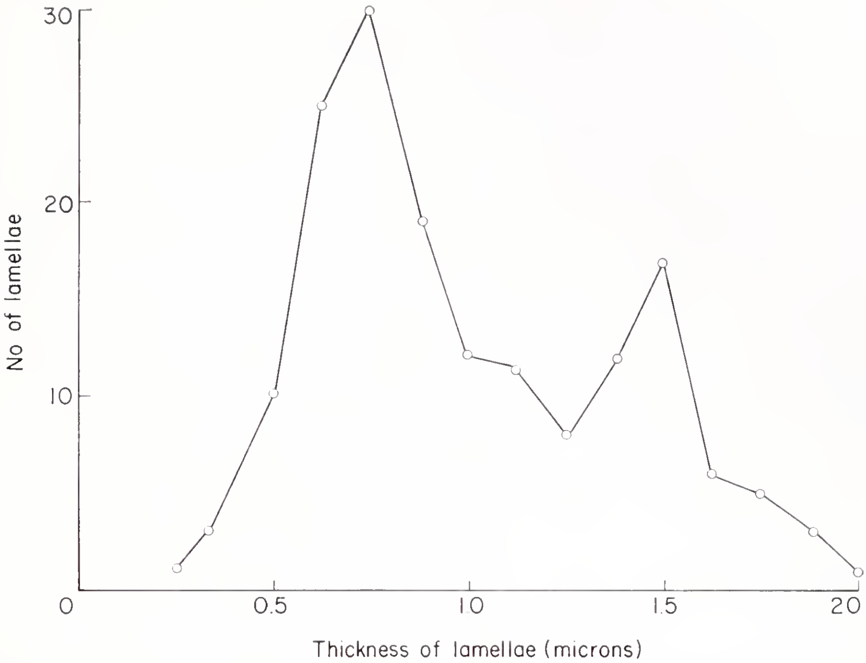

FIGURE 15. Plot of thickness of lamellae in fractured shell of adult *Mytilus edulis*.

Schaadt, and Peters, 1974) creates spaces among them, thereby weakening the shell sufficiently for the radula to dislodge and remove the units. A similar type of dissolution of the shell of *Mytilus edulis* is caused by secretions from the shell burrowing polychaete, *Polydora websteri* (Zottoli and Carriker, 1974). If prisms occur parrallel to the surface of the borehole (much as in Fig. 5d, Zottoli and Carriker, 1974), the radula breaks out long fragments of shell (Fig. 1, this paper); on the other hand, if prisms occur more or less at right angles to the surface of the borehole (much as in Fig. 13, Carriker, Schaadt, and Peters, 1974), the radula tends to cut out mostly short pieces of shell units (Fig. 5, this paper).

The mucoid material which blankets shell fragments on their journey from the buccal cavity to the stomach and out the intestine probably insulates them against further corrosion from acid juices in the stomach. More importantly, however, the mucoid envelope must also serve to insulate sharp edges of shell fragments which might otherwise lacerate the delicate ciliated epithelium of the alimentary canal. Since the mucoid material undoubtedly also contains a large proportion of viscid secretion from the accessory boring organ, it would appear that a secondary function of the secretion from this gland is to contribute to clothing of sharp shell fragments on their way down the digestive tract.

It is inviting to speculate whether hole boring contributes calcium carbonate to the calcium anabolism of boring snails. Observations reported in this paper, however, suggest that very little dissolution of the shell occurs in the stomach. The question could be settled by incorporating a calcium isotope in wafers of ground shell rasped by snails in devices patterned after the valve model of Carriker and Van Zandt (1972b). The isotope could then be traced through the organ systems of the snail. Further support for the conjecture that the snail does not utilize its shell chips to any extent in supplementing its calcium needs comes from the fact that shell pellets remain only briefly in the stomach.

Why shell rasped from the borehole is swallowed rather than pushed out by the propodium in its sweep across the surface of the borehole after each rasping period (Carriker and Van Zandt, 1972b) may be explained on morphological grounds. Simply stated, the effective stroke of the radula over the substratum is from front to back (Carriker, Schaadt, and Peters, 1974), and the load gathered on each stroke is dumped onto the esophageal valve and by suction from the anterior esophagus is carried immediately into the esophagus. Furthermore, the valve of Leiblein present in the esophagus of snails with long proboscides, like those of the Muricacea and Buccinacea, is thought to prevent regurgitation of food from the more posterior parts of the gut during elongation of the proboscis (Fretter and Graham, 1962), an arrangement which would tend to deter necessary reversal of flow in the anterior alimentary canal required for oral discharge of shell raspings.

Although the accessory boring organ determines the shape and size of the borehole through dissolution of shell (Carriker and Van Zandt, 1972a), no one has calculated how much dissolved calcium or other components of shell are absorbed into the body of the snail through the accessory boring organ during the process of shell boring. It is likely that a substantial amount of the dissolved shell mixes with the secretion of the accessory boring organ and, with shell chips broken off by the radula, finds its way down the alimentary canal.

This study graphically demonstrated the extraordinary capacity of boring gastropods to dismantle the intricately architectured mineralized valves of prey in search for food. The process plainly combines mechanical-chemical functions (Carriker and Van Zandt, 1972b), and effective removal of shell is impossible by radular or chemical action alone (Carriker, Person, Libbin, and Van Zandt, 1972; Carriker and Van Zandt, 1972b). Undoubtedly, rasping came first in the evolution of the group because the radula appeared early as a tool in food procurement. The next step, of rasping the shell of prey as patches of shell dissolving glandular tissue evolved to facilitate penetration, would be a logical one—and with it the act of swallowing shell chips simply as an available, functional, and economical avenue for their discharge!

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SUMMARY

1. Observations are reported on the ultrastructure of shell material rasped by *Urosalpinx cinerea follyensis* Baker from boreholes in the valves of *Mytilus edulis* Linné and transported normally to the stomach through the buccal cavity and esophagus. Duration of the period of chemical activity by the accessory boring organ and rasping by the radula were determined with a valve model. Pellets of shell raspings were removed from the stomach and, after fracturing to reveal the interior, and coating with metal, were studied with the scanning electron microscope. Shell raspings were compared with prisms and lamellae in fracture surfaces of normal shell of *M. edulis* and shell etched with ethylenediamine and sodium hypochlorite to reveal the form of shell units clearly.

2. The study provided ultrastructural evidence for the first time that *Urosalpinx cinerea* swallows shell rasped from the borehole during penetration of prey. Both prisms and lamellae were identified in the pellets removed from the stomach. Noticeable dissolution of the organic matrix, and to some extent also of the mineral portion, of prisms was evident, features which facilitate removal of shell by the snail during rasping.

3. If the long axis of prisms occurs parallel to the surface of the borehole, the radula tends to rasp out long fragments of shell; if prisms are placed at right angles to the surface, the radula breaks prisms into small pieces.

4. The envelope of mucoid material which coats pellets undoubtedly reduces, or prevents, laceration of the epithelium of the alimentary canal as shell fragments pass down the tract.

5. A gross approximation of the percentage of shell in the borehole which is rasped and swallowed during the process of hole boring is 14%.

LITERATURE CITED

Carriker, M. R., 1969. Excavation of boreholes by the gastropod, *Urosalpinx:* an analysis by light and scanning electron microscopy. *Am. Zool.,* **9**: 917–933.

Carriker, M. R., and D. Van Zandt, 1972a. Regeneration of the accessory boring organ of muricid gastropods after excision. *Trans. Am. Microsc. Soc.,* **91**: 455–466.

Carriker, M. R., and D. Van Zandt, 1972b. Predatory behavior of a shell-boring muricid gastropod. Pages 157–244 in H. E. Winn and B. L. Olla, Eds., *Behavior of marine animals: current perspectives in research, Vol. 1, invertebrates.* Plenum Publishing Corp., New York.

Carriker, M. R., P. Person, R. Libbin, and D. Van Zandt, 1972. Regeneration of the proboscis of muricid gastropods after amputation, with emphasis on the radula and cartilages. *Biol. Bull.,* **143**: 317–331.

Carriker, M. R., J. G. Schaadt, and V. Peters, 1974. Analysis by slow-motion picture photography and scanning electron microscopy of radular function in *Urosalpinx cinerea follyensis* (Muricidae, Gastropoda) during shell penetration. *Mar. Biol.,* **25**: 63–76.

Fretter, V., and A. Graham, 1962. *British prosobranch molluscs, their functional anatomy and ecology.* The Ray Society, London, 755 pp.

Graham, A., 1941. The esophagus of the stenoglossan prosobranchs. *Proc. R. Soc. Edin. Sect. B*, **61**: 1–23.

Graham, A., 1953. Form and function in the molluscs. *Proc. Linn. Soc. London,* **164**: 213–217.

Mutvei, H., 1970. Ultrastructure of the mineral and organic components of molluscan nacreous layers. *Biomineralization Res. Rept.,* **2**: 48–72.

Mutvei, H., 1972. Ultrastructural studies on cephalopod shells. Part 1. The septa and siphonal tube in *Nautilus. Bull. Geol. Inst. Univ. Ups.* **8**: 237–261.

Travis, D. F., and M. Gonsalves, 1969. Comparative ultrastructure and organization of the prismatic region of two bivalves and its possible relation to the chemical mechanism of boring. *Am. Zool.*, **9**: 635–661.

Zapanta, R. R., and O. R. Trautz, 1961. The separation of the mineral phase in mineralized tissues. *J. Dent. Res.*, **40**: 702.

Ziegelmeier, E., 1954. Beobachtungen über den Nahrungserwerb bei der Naticide *Lunatia nitida* Donovan (Gastrophoda Prosobranchia). *Helgol. Wiss. Meeresunters.*, **5**: 1–33.

Zottoli, R. A., and M. R. Carriker, 1974. Burrow morphology, tube formation, and microarchitecture of shell dissolution by the spionid polychaete *Polydora websteri*. *Mar. Biol.*, **27**: 307–316.

Reference: *Biol. Bull.*, **152**: 337–350. (June, 1977)

NUTRITIONAL REQUIREMENTS OF THE WATER FLEA *MOINA MACROCOPA*

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Herbivorous crustaceans comprise a significant proportion of filter-feeders; among them, Cladocera are found in almost all types of fresh waters. Although these abundant animals, known as water fleas, have been used extensively for a variety of research interests, little is known about their nutritional requirements.

Water fleas have been easily grown for a few generations on bacteria and algae, but maintaining high yields and continued fertility for many generations under agnotobiotic conditions is difficult. Rapid changes in microflora with attendant accumulation of metabolic products are often unfavorable and common in static cultures. A flowing system fed with algae grown in a chemostat offers better chances for stability and continued fertility (M. C. Glendening, University of Washington, personal communication).

The goal of sustained cultures can also be attained by growing Cladocera aseptically (D'Agostino and Provasoli, 1970; Murphy, 1970) on one or more algal species in media supplemented by organic nutrients and vitamin mixtures. Obviously, synxenic cultures, although successful, do not allow identification of nutritional factors for continued fertility.

Details on the composition and preparation of the artificial particulate media which support over 200 parthenogenetic generations of *Moina macrocopa americana* are given elsewhere (Conklin and Provasoli, in preparation). This study reports the nutritional requirements of *Moina* revealed during the substitution of crude nutrients by chemically defined constituents. Since the final medium has still one undefined component (liver infusion), definition of the minimal nutritional requirements has to be postponed.

MATERIALS AND METHODS

Work on *Moina* was initiated after failure to grow *Daphina magna* on artificial media beyond one or two generations. Dr. James Murphy of the Rockefeller University suggested that *Moina macrocopa americana* might be more suitable, since it could be grown indefinitely on only one algal species as food organism; the other Crustacea tried, including *Daphnia,* required two algae (Murphy, 1970).

At first the inoculum was taken from maintenance cultures of *Moina* fed on *Chlamydomonas reinhardii* synxenically grown in a modified *Daphnia* medium (*i.e.*, the *mineral* part of the basal medium, Table I) or in DA medium (D'Agostino and Provasoli, 1970). Single young females served as inoculum after being freed of the accompanying algae by 7–10 serial transfers in 10 ml of mineral base, then fed overnight on the same medium with added particles of albumin-starch co-gel (SAgel, Table I) to cleanse their gut from algae.

TABLE 1

Artificial media

Common balal medium (*per cent w or v/v*)

KCl, 3 mg; $MgSO_4 \cdot 7\ H_2O$, 4 mg; Ca (as Cl^-), 2 mg; K_3PO_4, 2 mg; $Na_2SiO_3 \cdot 9\ H_2O$, 2 mg; metal mix PII, 1 ml (1 ml contains Na_2EDTA, 1 mg; Fe, 0.01 mg; B, 0.2 mg; Mn, 0.04 mg; Zn, 0.005 mg; Co, 0.001 mg); Fe (as $(NH_4)_2$ H citrate), 0.05 mg; glycylglycine, 50 mg, pH 8.0 (TRIS buffer (Sigma Co.) is toxic for *Artemia*, *Daphnia* and *Moina* at 50 mg%. TES buffer (Sigma Co.) is nontoxic at 100 mg% for *Moina*); nucleic acid mix V, 2 ml (1 ml contains adenylic acid, 20 mg; guanylic acid, 10 mg; cytidylic acid, 10 mg; thymidine, 10 mg; dissolve in alkali, adjust to pH 8.0); DF 2, 1 ml (1 ml contains TWEEN 60, 2 mg; TWEEN 80, 2 mg; rutin, 0.5 mg; oxbile extract (Nutritional Biochem Co.), 1 mg.; disperse and emulsify components; adjust to pH 8.0); Cholesterol, 0.6 mg (dissolved in 95% ethanol, squirted into boiling water, ethanol boiled off; forms fine crystalline precipitate); amino acids mix H1, 1 ml (1 ml contains L-isoleucine, 10 mg; L-lysine HCl, L-glutamic acid, L-histidine base, L-threonine, L-methionine, L-leucine, L-valine, L-proline, 1 mg each; L-arginine base, L-tyrosine, L-serine, glycine, L-tryptophane, 0.5 mg each); vitamin mix M1B, 1 ml (1 ml contains thiamine HCl, 0.5 mg; nicotinamide, 1.5 mg; pyridoxine HCl, 0.2 mg; biotin, 0.06 mg; putrescine·2 HCl, 0.1 mg; Vitamin B_{12}, 0.002 mg; choline H_2 citrate, 0.2 mg; riboflavin, 0.2 mg; folic acid, 0.1 mg; Ca pantothenate, 4 mg); Liver infusion L 25 (Oxoid, Flow Labs, Rockville, Md.), 70 mg (does not dissolve completely; upon autoclaving in medium forms a brown precipitate essential for growth). Adjust pH of basal medium to pH 7.6–7.8.

Particulate additions (*per cent*)

Essentially the particles are formed by dissolving or emulsifying the components in water; coagulating the mix by autoclaving; homogenizing the coagulum to produce fine particles (2–20 μm); autoclaving the particle slurry and homogenizing it again. Homogenization of particles containing lipids is done under flowing nitrogen; the mix is stored in well closed containers with N; add 1 drop preservative (O-fluorotoluene, 1 part; 1.2 dichloroethane, 1 part; 1 chlorobutane, 3 parts; Eastman Organic Chemicals) and store in refrigerator; active for 1–2 months. [Details for preparation of particles are given in Conklin and Provasoli, in preparation.]

E medium: Basal medium + trigel particles 2 ml [supplying egg albumin, 15 mg; rice starch, 10 mg; dry beef serum, 5 mg] + egg particles, 0.2 ml [supplying egg yolk, 10 mg; vitamin E (type II), 2 mg; calciferol, 0.5 mg].

FP medium: Basal medium + trigel particles 2 ml (as above) + FP particles 1 ml [supplying albumin fraction V, 4.5 mg; vitamin E (type II), 3 mg; egg lecithin, 1.5 mg; calciferol, 0.75 mg].

F1 medium: Basal medium + SA gel particles 2 ml [supplying egg albumin, 15 mg; rice starch, 10 mg] + FV particles 1 ml [supplying albumin fraction V, 6 mg; egg lecithin, 1.5 mg; BHT (butylated hydroxytoluene), 1 mg; calciferol, 1 mg; β-carotene, 0.5 mg; *dl*-α-tocopherol, 2 mg; linolenic acid, 1.5 mg; linoleic acid, 1 mg; palmitic acid, 1 mg; oleic acid, 0.5 mg].

This time-consuming operation was obviated when *Moina* was maintained on a medium containing egg-yolk particles and, later, on the more nearly defined artifical medium. In these media parthenogenetic females produced a brood of 4–6 newborn every other day.

For uniformity of inoculum, a few fertile females without visible eggs were transferred into tubes of artificial media; within the second day they produced a brood. The females were transferred to new tubes leaving behind their brood. Two days later these newborn became young females and were transferred singly to the experimental test tubes (10 ml, 25 × 120 mm). To insure rapid and uniform growth, the tubes were shaken daily on a Vortex Genie (Scientific Industries, Inc.) to resuspend the fine particulates. The animals were not harmed by shaking; nevertheless, each tube was inspected to insure that no animal was trapped on the side of the test tube above the medium.

The test tubes were incubated at 25° C in very dim continuous light to avoid rapid destruction of light-sensitive vitamins (riboflavin and folic acid).

To reduce inherent variability of the inoculum (consisting of individuals born within 2 days, in a life-cycle lasting only 4–7 days), day 1 of each tube was the day in which the animal released the first brood. To score the effectiveness of each nutritional variable, the animals were killed on the seventh day by adding one drop of 1 ml % formalin and counted under the dissecting microscope in a plastic Bogarov counting tray (Wickstead, 1965).

Ten tubes of each experimental variable were inoculated with one juvenile; of these, seven tubes were counted on the seventh day and the count averaged; the remaining three tubes were killed and counted when the food particles had been almost completely exhausted (80–85% transmittance). The seven-day period allowed the first two broods of the inoculated female to reach maturity and to produce their first brood on day 7. The combined three generations growing in each test tube consisted of 13–19 adult females and 90–110 newborn and juveniles.

This procedure led, nonetheless, to variability in counts. Variations due to media preparation could be excluded, since all the variable consitutents of one experiment were added to portions of a common basal medium before being dispensed.

Some variability could be ascribed to the inoculum. Virgin females were not used to prepare inocula; hence, progeny could range from the first to fourth brood. Murphy and Davidoff (1972) have shown that early broods are less exacting in nutritional requirements, perhaps because of receiving more nutrients from the mother. However, considerable variability was also noted in this study among individuals in the same brood.

A "biological conditioning" occurred in nutritionally adequate media. Newborn, inoculated singly in a new test tube, generally took seven days from birth to become adult females and produce the first brood; these first brood individuals reached maturity and produced the first brood in only four days. The size of the brood also varied: the original female produced 4–6 newborn, which in turn produced a larger brood (6–8); the second and third brood of the original female might also be 6–8.

Choice of the seventh day for counting was arrived at empirically. Nutritionally complete media on day 7 gave counts ranging from 90–120 individuals, while counts from media lacking an essential nutrient were about 10–30; counts corresponded well to changes in concentration of essential nutrients. This indicated that the nutritional carry-over from the original juvenile female had been largely offset by the effect of the medium on her and her progenies' time to adulthood and brood size. Serial transfers would have provided a complete definition of the requirements if the medium was completely defined. Differences within a nutritional variable were reduced by averaging the counts of seven test tubes; however, if the range was large, the variable was retested. The remaining three replicate test tubes were allowed to go to exhaustion of the particles; such tubes served as extra controls to gauge whether the large variability in the seven counts was an artifact due to the compounded effects of a delay of a few hours in the hatching of the F_1 and F_2 broods.

The artificial media were biphasic, and consisted of a liquid and a particulate phase (Table I).

RESULTS

Initial attempts to grow a freshwater parthenogenetic cladoceran were as noted, with *Daphnia magna.* Profiting from experience, we added to the mineral base for *Daphnia* (D'Agostino and Provasoli, 1970) the components of the defined medium for *Artemia* (Provasoli and D'Agostino, 1969), *i.e.*, B vitamins, nucleic acids, cholesterol and starch-protein particles. Only occasional adults were obtained at first; after arriving at more suitable concentrations of the components, adults were regularly obtained but produced fertile eggs only sporadically. Incorporation of beef serum into the starch-protein particles (tri-gel, Table I) resulted in fertile adults but most nauplii never reached adulthood. Upon the advice of Dr. Murphy the trigel medium was tried on *Moina;* two fertile generations and, occasionally, a third generation were obtained.

The manifest improvement of fertility by serum addition focused attention on lipids. Coagulated egg-yolk particles improved growth and survival of newborn and the brood size of *Moina* and allowed an occasional second generation of *Daphnia.* Following the lead of Viehoever and Cohen (1938), vitamin E was incorporated in the egg yolk particles; six fertile generations of *Moina* ensued. Finally addition of vitamin D led to continuous growth of *Moina* (>200 generations). Since only six consecutive generations of *Daphnia* could be obtained in the best medium, *Daphnia* was set aside.

The egg medium (Table I) was used from then on for maintenance and for production of inocula and served as a yardstick in experiments leading to more defined media.

Since the vitamin E added to egg yolk (Type II of Sigma Chemical Co.) is a mixture of 50% vegetable oils and 50% dl-α-tocopherol, we sought to replace it with its components. But combinations with several vegetable oils and dl-α-tocopherol were inferior to "type-II" concentrate. Attempts to replace the egg yolk particles by incorporating vitamin E type II and vitamin D into the trigel failed but led to resolution of the impasse. The lipid fraction proved too large to be entrapped during coagulation by the albumin and serum proteins; it leached into the medium. Better retention of the lipids was sought by employing more albumin in the trigel, but still the oils separated. Finally the lipids were incorporated into a separate particle, exploiting the fat-binding properties of albumin fraction V. This new particle (FP, Table I) replaced egg yolk in maintaining viability of successive generations but resulted in smaller broods and longer time to adulthood.

The decision to incorporate all the lipid factors in one type of particle and to remove the serum from the tri-gel proved decisive. We regained ability to experiment with the starch : protein : lipid ratios, and to improve the lipid components and ratios within a separate particle. Evenutally, with incorporation of lecithin and a suitable fatty-acid mixture, the new lipid-albumin particle (FV, Table I) replaced completely serum and egg yolk.

The nearly-defined medium (F1) has minerals, trace metals, amino acids, nucleic acids, B vitamins, and liver infusion in the liquid phase; an emulsion of TWEENS and bile salts (DF_2); a fine suspension of cholesterol crystals; and two larger particles (2–20 μm), the protein-starch co-gel (SA) and the complex FV particle containing egg lecithin, vitamin A, D, and E, four fatty acids and an antioxidant emulsified and entrapped in the heat-coagulated albumin fraction. The last undefined component of the medium is the Oxoid liver infusion which improves growth and fertility.

The results presented here derive from experiments aimed at replacing efficiently the unknowns that contributed to high, continuous reproduction. Experiments to define the minimal essential requirements were postponed because complete replacement of the liver infusion was not achieved. However, the data in the tables were obtained with the nearly defined F1 medium.

The mineral base seems quite adequate. Sporadic changes in the concentrations of the major elements and replacement with other trace metal mixtures effected no substantial improvement.

The vitamin requirements were analyzed using the egg particles and, later, the FV particles (Table II). The results were quite similar: thiamine, nicotinamide, pyridoxine, and pantothenic acid were clearly essential. Omission of folic acid and riboflavin permitted some growth and low fertiliy—as expected—for the medium had 70 mg% of liver infusion which is notoriously rich in these vitamins. They were needed, however, because addition of folic acid and riboflavin increased fertility and partly replaced, the liver infusion in medium F1 when increased above the levels in the vitamin mix. With the present media, no clear need was found for *p*-aminobenzoic, lipoic, and ascorbic acids, inositol and carnitine. Biotin, choline, cyanocobalamin, and putrescine were retained in the vitamin mixture, because they had shown some activity in several experiments, and for safety when

TABLE II

Dose response of water-soluble vitamins in F1 medium.

Vitamin (mg%)		Production in 7 days*	Vitamin (mg%)		Production in 7 days*
Thiamine HCl	0	8	Pyridoxine HCl	0	15
	0.4	86		0.2	90
	0.5	92		0.3	102
	0.6	98		0.4	104
	0.8	90		0.5	104
	1.0	80		0.6	76
Nicotinamide	0	21	Ca Pantothenate	0	34
	0.5	44		0.5	73
	1.0	58		2.0	100
	1.5	80		3.0	97
	2.0	95		4.0	107
	2.5	107		5.0	96
	3.0	88			

* Number of individuals produced by one female in 10 ml of medium at day 7.

replacing the liver infusion. Some vitamins had sharp optimal zones and inhibition at higher concentrations.

Nucleic acids were indispensable for fertility; their omission permitted the growth to adult of the original inoculum; but no progeny were produced, although the adult female remained alive up to three weeks. Adenylic acid is indispensable, not replaceable by guanylic acid, and seems to constitute the bulk of the nucleic-acid requirement. Best results were obtained with a mixture of adenylic, guanylic, cytidylic acids, and thymidine at a 2× concentration (Table III); lower or higher concentrations were less effective. Supplementation with various amounts of uridylic acid improved neither growth nor fertility. Adenine could not replace adenylic acid.

Cholesterol was indispensable and was supplied as a crystalline slurry (an ethanol solution injected in water) or as an emulsion with Tweens and bile acids (ethanol solution added to DF_2; see Table I). The optimal concentration was 0.6 mg%; 0.7 and 0.8 were still good but 0.9 mg and above was inhibitory; no attempts were made to replace it with the phytosterols used by insects.

A vitamin D requirement emerged in early experiments, when its incorporation into the egg-yolk particles permitted continuous generations in *Moina*. When omitted from the FV particles fertility of the F_2 was drastically lowered as shown by the small difference between the counts at day 7 and the counts after exhaustion

TABLE III

Effect of purine and pyrimidine nucleotides in F1 medium.

Nucleic acids (mg %)		Production in 7 days*
1. None		None
2. adenylic	40	15
3. adenylic	40	64
guanylic	20	
cytidylic	20	
4. adenylic	40	51
guanylic	20	
thymidine	20	
5. adenylic	40	42
cytidylic	20	
thymidine	20	
6. guanylic	20	14
cytidylic	20	
thymidine	20	
7. guanylic	30	23
cytidylic	30	
thymidine	30	
8. Nucleic mix V [adenylic 20, guanylic 10, cytidylic 10, thimidine 10]	1 ×	53
9. Nucleic mix V	2 ×	97
10. Nucleic mix V	3 ×	87
11. Nucleic mix V	4 ×	41

* Number of individuals produced by one female in 10 ml of medium at day 7.

TABLE IV

Production of Moina *in absence of fat-soluble vitamins and fatty acids in F1 medium.*

	Production in 7 days*	Final count (particles consumed)
Vitamin omitted		
vitamin A (or β-carotene)	69	159
vitamin D_2 (calciferol)	57	89
vitamin E (dl-α tocopherol)	41	79
None omitted	103	239
Fatty acids omitted		
palmitic acid	20	50
oleic acid	72	201
linoleic acid	87	168
linolenic acid	72	147
None omitted	98	251

* Number of individuals produced by one female in 10 ml of medium at day 7.

of the particles, *i.e.,* all particulate food was consumed but without increasing fertility (Table IV).

Omission of *dl-α* tocopherol acted similarly. Omission of β-carotene permitted lower but continued fertility, *i.e.,* higher counts at total consumption of particulate food (Table IV). To test whether vitamin E was an essential nutrient or its effect on growth and fertility was due to its antioxidant properties, the antioxidant BHT (butylated hydroxytoluene) was tried as a replacement for vitamin E (Table V). From the interchangeability of BHT and tocopherol, it was evident that the main action of tocopherol was that of antioxidant. Even though BHT at 2 mg% allowed excellent growth and fertility despite total absence of tocopherol,

TABLE V

Production of Moina *in F1 medium at various levels of antioxidants.*

(mg %) Vitamin E*/BHT	Production in 7 days**	Final count (particles consumed)
2.0/4.0	59	96
2.0/3.0	82	165
2.0/2.0	94	186
2.0/1.0	105	214
2.0/0.0	104	220
1.5/2.0	83	185
1.5/1.5	92	200
1.5/1.0	117	227
0.8/3.0	95	178
0.8/2.3	102	171
0.8/1.5	91	193
0/4.0	0	0
0/3.0	39	88
0/2.0	67	175

* 1 ml of tocopherol was assumed to equal 1 g.

** Number of individuals produced by one female in 10 ml of medium at day 7.

tocopherol alone or in combination elicited more growth and production of young, indicating that it might also be an indispensable nutrient.

Fatty acids were essential for growth and fertility as noted in the original substitution of serum and egg-yolk. Omission of palmitic acid curtailed fertility drastically; it was the only fatty acid which elicited linearly increased growth with increased concentration; production in 7 days at 0.5 mg% was 76 individuals; at 1 mg% 120 and 140 individuals at 1.5 mg%. Omission of linolenic seemed to affect fertility of the F_2 more than linoleic; oleic may be dispensable in the presence of other unsaturated fatty acids (Table IV).

Discussion

The main achievement of this study was the formulation of a nearly-defined medium which permits rapid growth and continued fertility of *Moina* for over 200 generations—a result reflecting recognition that fatty acids, phosphatides and vitamins A, E, D are essential for sustained fertility and that the way of presenting the needed nutrients is in itself crucial for rapid growth.

Work on *Artemia salina* (Provasoli and D'Agostino, 1969) had shown that particulate media solve the impasse of an inefficient uptake of solutes by supplying the bulk nutrients (protein, carbohydrate and lipids) in particulate form, thus complying with, and exploiting, the great ability of filter feeders to gather particles. The suspicion that lipids might be indispensable for fertility led to the use of coagulated egg-yolk and later to the formulation of a repeatable and more chemically defined particle which supplies the lipids in a readily acceptable form.

The present medium is defined except for liver infusion; replacement of this component, notoriously rich in growth factors, is in progress. Consequently, we could define only those needs which were demonstrable above the levels in liver infusion. Hence, while a clear need for thiamin, nicotinamide, pyridoxine and pantothenic acid could be demonstrated, the omission of folic acid and riboflavin reduced fertility noticeably, but not completely. A need for biotin could not be shown; it is needed by most insects, by *Artemia* and is probably needed by *Moina* also.

These seven vitamins are probably needed by other Cladocera; Murphy's success in rearing several species of Cladocera in monoxenic cultures with *Chlamydomonas reinhardii* depended upon increasing the concentrations of several B vitamins, among them choline and inositol. Under Murphy's conditions, addition of inositol in biweekly pulses, at a concentration which was inhibitory if added continuously, was essential to obtain several generations from newborn of the fourth and fifth orthoclone, increasing life-span and brood size (Murphy and Davidoff, 1972). Under our conditions we could not detect response to inositol or choline (which like inositol is needed by several insects). Since our media contain lecithin, either choline is not needed or the need is satisfied by 1 mg% lecithin.

Several B vitamins became inhibitory at supraoptimal concentrations, particularly folic acid and riboflavin. Excess vitamins were also inhibitory to several insects (Akov, 1962; Vanderzant, 1963; Horie, Watanabe and Ito, 1966), indicating that the widespread notion that overdoses of B vitamins are harmless is unjustified.

Nucleic acids are needed for fertility in *Moina*. The need for adenylic acid exceeds the requirement for other nucleotides. *Artemia* also requires nucleotides, but they are already needed for growth from nauplii to adults; its requirement for adenylic acid is much higher than *Moina*, and *Artemia* also requires uridylic acid. While *Moina* and *Artemia* may be able to synthesize nucleotides, the concentrations required indicate that synthesis of nucleotides cannot keep pace with rapid growth.

Adenylic acid is apparently needed for ATP production, since Hernandorena (1974, 1975, 1976) found that the concentrations required by *Artemia* varied with concentrations of calorigenic nutrients (carbohydrates and fats) and that variations in temperature and salinity had opposite effects on the requirements for adenylic, protein and energetic nutrients. Most insects synthesize nucleic acids; but several Diptera, two beetles and the moth *Plodia* (Morère, 1974) need an exogenous source, perhaps because the rate of synthesis may be too low. In Diptera it has to sustain the thousand-fold increase in larval size which normally occurs in four days (House, 1972). In insects the need for nucleotides is satisfied by RNA and not by DNA; only *Agria affinis* utilizes both (House, 1964). While the house fly and *Drosophila* can utilize purine bases, adenine being the most important, *Agria affinis* like *Moina* and *Artemia* cannot utilize the purine and pyrimidine bases—it needs nucleotides.

A need for exogenous sterol is characteristic of insects and is met by cholesterol for all except *Drosophila palea*, which is monophagous on a cactus and needs schottenol or other Δ7 sterols. Stigmasterol, β-sitosterol and other phytosterols are equally well utilized by several phytophagous and omnivorous insects. A requirement for sterols may be common to all Arthropoda since ^{14}C-labeled precursors were not incorporated into sterols by the crab *Cancer* (van den Ord, 1964), the crayfish *Astacus* (Zandee, 1966), *Artemia* (Teshima and Kanazawa, 1971b), and prawn *Penaeus* and lobsters *Panulirus* (Teshima and Kanazawa, 1971c) and *Homarus* (Zandee, 1967), the spider *Avicularia* and the millipede *Graphidostreptus* (Zandee, 1967). *Artemia* and *Moina* need cholesterol, which seems to be better utilized than other sterols. Less effective utilization of phytosterols was also found for *Penaeus* (Kanazawa, Tanaka, Teshima and Kashiwada, 1971a). Teshima and Kanazawa (1971d) found that regardless of the sterol supplied (ergosterol, stigmasterol, beta-sitosterol and campesterol), *Artemia* contained only cholesterol; the prawn *Penaeus* behaved similarly (Kanazawa, Tanaka, Teshima and Kashiwada, 1971b). Cholesterol is apparently the only sterol found in several Crustacea (Teshima and Kanazawa, 1971a), indicating that the ability to convert dietary sterols into cholesterol may be wide-spread in Crustacea. Exogenous isotopically-labeled cholesterol contributed to the radioactivity of ecdysones in insects (Robbins, Kaplanis, Svoboda and Thompson, 1971) and of progesterone, androsterone and corticosterone in the hepatopancreas, ovaries and blood of the spiny lobster, *Panulirus* (Kanzawa and Teshima, 1971).

Vitamin D_2, calciferol, one of the isomers produced by UV-irradiation of ergosterol, is needed by *Moina* for fertility. *Moina* and many insects (Dadd, 1973) cannot use calciferol as a substitute for cholesterol. Calciferol, besides cholesterol, is needed to maintain fecundity, indicating that *Moina* presumably cannot synthesize it from cholesterol. A mixture (1 : 1) of ergosterol and olive oil absorbed on defatted yeast cells induced, in bacterized cultures of *Moina rectirostris*, production of 30% males and ephippial eggs (von Dehn, 1955).

Ergosterol is present in several species of phytoplankters and could be transformed into calciferol by irradiation, hence become available to Crustacea, even if it is not directly synthesized by the algae. The need for calciferol or its hydroxylated hormonal forms in insects might have been missed, because it was tested only as a substitute for cholesterol and because only recently has adequate attention been given to the requirements for continuous reproduction.

Fertility in *Moina* depends also on exogenous β-carotene or retinol and α-tocopherol. A vitamin E requirement for repairing ovarian disfunction and restoring fertility was first postulated by Viehoever and Cohen (1938), who suggested *Daphnia magna* for assay of the vitamin. The water-soluble sodium salt of dl-α-tocopherol phosphate lengthened the life of *Daphnia longispina* grown on yeast (Fluckinger and Fluck, 1950). Vitamin E was included early in insect diets to protect and delay oxidation of fatty acids, following the lead of Fraenkel and Blewett (1946) and Vanderzant, Kerur and Reiser (1957), but it was not recognized as a specific fertility factor until Chumakova (1962) reported that vitamin E was necessary for egg production of the beetle *Cryptolemus*. It is also necessary for viable sperms in the cricket *Acheta* (Meike and McFarlane, 1965), for a viable offspring in the parasitoid *Agria affinis* (House, 1966), for pupal development in the beetle *Oryzaephilus* (Davis, 1967), for the egg hatchability in the mite *Tetranychus* (Ekka, Rodriguez and Davis, 1971) and for female fecundity in the moth *Plodia* (Morère, 1971a). The concentration of tocopherol added to insect diets varied from 1 mg% to 200 mg% (for *Plodia*).

Apparently only Fraenkel and Blewett (1946) tried to discriminate between the antioxidant nonspecific effect and a specific effect on fertility. Though the antioxidant stabilization of fatty acids by tocopherol could be replaced by dietary ascorbic acid and ethyl or propyl gallate, they concluded that α-tocopherol has an independent growth promoting effect. This seems to apply to *Moina* for which good fertility for three generations was obtained with 2 mg% BHT in the absence of tocopherol, but addition of tocopherol to BHT resulted always in larger broods and better viability. Despite the pioneering work of Frankel, failure to detect the need for tocopherol in more insects is probably due to contamination of the lipids available in the early studies and to restriction of the nutritional analysis to one generation.

β-carotene and vitamin A are apparently essential for normal pigmentation in locusts (Dadd, 1961) and grasshoppers (Nayar, 1964) and synthesis of visual pigments, lack of which causes a loss of visual sensitivity (Goldsmith, Barker and Cohen, 1964; Zimmerman and Goldsmith, 1971). A direct effect on growth has been seldom observed; vitamin A improved larval growth in *Agria* (House, 1966) and carotene in locusts (Dadd, 1961) and *Plodia* (Morère, 1971b). Omission of carotene from the diet lowered the fertility of *Moina* noticeably but less than the omission of vitamin D and E and is apparently the first report for Crustacea. The claim of Dutrieu (1959) that carotenoids are essential for *Artemia* seems to be supported experimentally, if as yet weakly.

Moina requires saturated and unsaturated fatty acids. Omission experiments indicated that palmitic, linolenic, and linoleic promote fertility. Only palmitic acid was indispensable; increased fertility was a direct function of increased palmitate; omission of the other fatty acids lowered fertility, but less sharply. After discovery that linoleic acid was needed for pupal eclosion and scale covering of wings in *Ephestia* (Fraenkel and Blewett, 1946), the dietary requirements for insects for saturated, mono- and poly-unsaturated fatty acids inspired a large literature (Dadd, 1973) and diverse requirements were found in different insect groups. Essentiality of polyunsaturated di- and trienoic C_{18} fatty acids is common in Lepidoptera, Orthoptera and Coleoptera; Diptera do not require them. Diptera may depend on a different lipid metabolism; ethanolamine phosphoglycerides and palmitoleic acid are their major lipids.

Isotopic studies indicate that insects synthesize saturated monoenoic fatty acids, and this seems to hold also for Crustacea (Zandee, 1967). Yet, especially in Diptera, palmitic and oleic acids are often needed for optimal growth. The importance of palmitic in *Moina* may be explained by the findings of Morris and Sargent (1973) that radioactive palmitic acid fed to, or injected in, Crustacea was used for biosynthesis of wax esters and high polyunsaturated fatty acids. Work with commercial diets for prawns and lobsters indicate that the ratio between $\omega 3$ and $\omega 6$ fatty acids may be an important factor.

It is evident then that Crustacea have requirements similar to insects, indicating that Arthropoda may have a rather homogeneous distinctive nutritional pattern. This similarity justifies, at least empirically in initial cultivation, the assumption that the general nutritional requirements of a group apply to a single species, but this cannot substitute for detailed studies.

Remarkable differences were found between *Artemia, Daphnia* and *Moina*. Even the initial attempts to grow *Daphnia* made it evident that the high starch/protein ratios (5:1) of *Artemia* did not suit *Daphnia* which preferred more protein. This was also true for *Moina*, for which the optimal range starch/protein lies between 1.5:1 and 0.5:1; within this range, the optimal ratio was influenced by the other components of the medium, particularly lipids. So far, egg albumin seems to satisfy the amino acid requirements of *Artemia* and *Moina*. For *Moina*, albumin fraction V inhibited above 8 mg% and could not replace egg albumin as the sole source of amino acids.

Nutrient ratios are well known to affect the efficiency of diets. Failure in attempts to shift the nutrition of invertebrates from natural food to artificial diet might be caused by improper ratios of nutrients, as well as by the selection of inadequate protein and lipid sources. Dietary efficiency is an important consideration for aqua-culture of shrimp, prawn and lobster. High protein diets seem to speed growth rates but also increase the cost of the diets, suggesting the need for more efficient carbohydrate:lipid:protein ratios. Extrapolation of our results indicated that the present diets may be deficient in nucleic acids and that means for preventing leaching of water soluble vitamins and minerals would greatly improve the efficiency of the commercial diets for animals which do not feed voraciously (Provasoli, 1976).

SUMMARY

1. *Moina macrocopa* was cultured aseptically for more than 200 parthogenetic generations in a nearly-defined medium without losing fertility.

2. A biphasic medium was used. The liquid phase supplied minerals, B-vita-

mins, amino acids, liver infusion and nucleic acids. The fine particulate phase consisted of egg albumin, albumin fraction V, starch and lipid factors.

3. The particulate phase was essential for rapid growth, taking advantage of the food gathering efficiency of filter feeders.

4. Developmental time, brood size and sustained fertility depended on calciferol, tocopherol and saturated and unsaturated fatty acids; B carotene or retinol favored fertility but might not be essential.

5. *Moina* was found to require cholesterol, nucleic caids, thiamine, nicotinamide, pyridoxine, Ca pantothenate and probably riboflavin and folic acid in the presence of liver infusion, the only undefined and required component of the medium.

6. Ratios and quantities of the nutrients were important for media efficiency: excess vitamins could be inhibitory and the best starch/protein ratio was in the range of 1.5 : 1 to 0.5 : 1.

7. The requirements of *Moina* were compared with the range of requirements of arthropods and found to be similar to the nutritional patterns of phytophagous insects.

LITERATURE CITED

AKOV, S., 1962. A qualitative and quantitative study of the nutritional requirements of *Aedes aegypti* larvae. *J. Insect Physiol.*, **8**: 319–335.

CHUMAKOVA, B. M., 1962. Significance of individual food components for the vital activity of mature predatory and parasitic insects. *Vop. Ekol. Kievsk.*, **8**: 133–134 (not seen in original, quoted from *Biol. Abstracts*, 45, 44, 502, 1964).

D'AGOSTINO, A. S., AND L. PROVASOLI, 1970. Dixenic culture of *Daphnia magna* Straus. *Biol. Bull.*, **139**: 485–494.

DADD, R. H., 1961. Observations on the effect of carotene on the growth and pigmentation of locusts. *Bull. Entomol. Res.*, **52**: 63–81.

DADD, R. H., 1973. Insect nutrition: current developments and metabolic implications. *Annu. Rev. Entomol.*, **18**: 381–420.

DAVIS, G. R. F., 1967. Effects of dietary lipids on survival and development of the sawtoothed grain beetle *Oryzaephilus surinamensis* (L.) (Coleoptera, Silvanidae). *Rev. Can. Biol.*, **26**: 119–124.

VON DEHN, M., 1955. Die Geschlechtsbestimmung der Daphniden. Die Bedeutung der Fettstoffe untersucht an *Moina rectirostris*. *Zool. Jahrb. Abt. Allg. Zool. Physiol. Tiere*, **65**: 334–356.

DUTRIEU, J., 1959. Les pigments *d'Artemia salina* L. Rapports entre la nature des pigments et le mode de reproduction des adultes. *C.R. Hebd. Seances Acad. Sci. Paris*, **248**: 2522–2524.

EKKA, I., J. G. RODRIGUEZ, AND D. L. DAVIS, 1971. Influence of dietary improvement on ovoposition and egg viability of the mite, *Tetranichus urticae*. *J. Insect Physiol.*, **17**: 1393–1399.

FLUCKINGER, E., AND R. FLUCK, 1950. Die Einfluss von Vitamin E auf *Daphnia longispina*. *Experientia*, **6**: 64.

FRAENKEL, G., AND M. BLEWETT, 1946. Linoleic acid, vitamin E and other fat soluble substances in the nutrition of certain insects (*Ephestia kuehniella, E. elutella, E. cautella* and *Plodia interpunctella* (Lep.). *J. Exp. Biol.*, **22**: 172–190.

GOLDSMITH, T. H., R. L. BARKER, AND C. F. COHEN, 1964. Sensitivity of visual receptors of carotenoid-depleted flies: a vitamin A deficiency in an invertebrate. *Science*, **146**: 65–67.

HERNANDORENA, A., 1974. Effects of salinity in the nutritional requirements of *Artemia salina*. *Biol. Bull.*, **146**: 236–248.

HERNANDORENA, A., 1975. Metabolic significance in nucleic acid metabolism and protein synthesis of dietary AMP requirement in *Artemia salina* L. *Biol. Bull.*, **148**: 416–428.

Hernandorena, A., 1976. Effects of temperature on the nutritional requirements of *Artemia salina*. *Biol. Bull.*, **151**: 314–321.

Horie, Y., K. Watanabe, and T. Ito, 1966. Nutrition of the silkworm, *Bombyx mori* XIV. Further studies on the requirements for B vitamins. *Bull. Seric. Exp. Stn. Tokyo.* **20**: 393–409.

House, H. L., 1964. Effects of dietetic nucleic acids and components on growth of *Agria affinis* (Fallen) (Diptera: Sarcophagidae). *Can. J. Zool.*, **42**: 801–806.

House, H. L., 1966. Effects of vitamin E and A on growth and development, and the necessity of vitamin E for reproduction in the parasitoid *Agria affinis* (Fallen) (Diptera: Sarcophagidae). *J. Insect Physiol.*, **12**: 409–417.

House, H. L., 1972. Insect nutrition. Pages 513–573 in R. N. Fiennes, Ed., *Biology of nutrition. Vol. 18.* Pergamon Press, Oxford.

Kanazawa, A., and S. Teshima, 1971. *In vivo* conversion of cholesterol to steroid hormones in the spiny lobster *Panulirus japonica*. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 891–898.

Kanazawa, A., N. Tanaka, S. Teshima and K. Kashiwada. 1971a. Nutritional requirements of Prawn. II. Requirements for sterols. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 211–215.

Kanazawa, A., N. Tanaka, S. Teshima, and K. Kashiwada, 1971b. Nutritional requirements of Prawn. III. Utilization of dietary sterols. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 1015–1019.

Meike, J. E. S., and J. E. McFarlane, 1965. The role of lipid in the nutrition of the house cricket *Acheta domesticus* L. (*Orthoptera*, Grillidae). *Can. J. Zool.*, **43**: 87–98.

Morère, J. L., 1971a. Remplacement de l'huile de germe de mais par des acides gras dans l'alimentation de type meridique de *Plodia interpunctella* (Hbn.) (Lepidoptère-Pyralidae) *C.R. Hebd. Seances Acad. Sci. Paris*, **272**: 133–136.

Morère, J. L., 1971b. Le carotène: substance indispensable pour la nutrition de *Plodia interpunctella* (Lepidioptère-Pyralidae). *C.R. Hebd. Seances Acad. Sci. Paris*, **272**: 2229–2231.

Morère, J. L., 1974. Nutrition d'une chenille de lepidoptère *Plodia interpunctella* (Hbn) (Lepidoptère-Pyraldiae) sur un milieu chimiquement défini. *Arch. Int. Physiol. Biochim.*, **82**: 625–630.

Morris, R. J., and J. R. Sargent, 1973. Studies on the lipid metabolism of some oceanic Crustacea. *Mar. Biol.*, **22**: 77–83.

Murphy, J. S., 1970. A general method for the monaxenic cultivation of the Daphnidae. *Biol. Bull.*, **139**: 321–332.

Murphy, J. S., and M. Davidoff, 1972. The result of improved nutrition on the Lansing effect in *Moina macrocopa*. *Biol. Bull.*, **142**: 302–309.

Nayar, J. K., 1964. The nutritional requirements of grasshoppers. 1. Rearing of the grasshopper *Melanoplus bivattatus* (Say) on a completely defined diet and some effects of different concentrations of B vitamin mixtures, linolenic acid and β-carotene. *Can. J. Zool.*, **42**: 11–22.

Provasoli, L., 1976. Nutritional aspects of crustacean aquaculture. Pages 13–21 in K. S. Price, M. N. Shaw, and K. S. Danberg, Eds., *Proc. First Int. Conf. Aquaculture Nutrition*, University of Delaware, Newark, Delaware.

Provasoli, L., and A. S. D'Agostino, 1969. Development of artificial media for *Artemia salina*. *Biol. Bull.*, **136**: 434–453.

Robbins, W. E., J. M. Kaplanis, J. A. Svoboda, and M. J. Thompson, 1971. Steroid metabolism in insects. *Annu. Rev. Entomol.*, **16**: 53–72.

Teshima, S., and A. Kanazawa, 1971a. Sterol compositions of marine crustaceans. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 63–67.

Teshima, S., and A. Kanazawa, 1971b. Utilization and biosynthesis of sterols in *Artemia salina*. *Bull. Jpn. Soc. Sci. Fish.*, **37**: 720–723.

Teshima, S., and A. Kanazawa, 1971c. Biosynthesis of sterols in the lobster *Panulirus japonica*, the prawn *Penaeus japonicus*, and the crab *Portunus trituberculatus*. *Comp. Biochem. Physiol.*, **38B**: 597–602.

Teshima, S., and A. Kanazawa, 1971d. Bioconversion of the dietary ergosterol to cholesterol in *Artemia salina*. *Comp. Biochem. Physiol.*, **38B**: 603–607.

Van den Ord, A., 1964. The absence of cholesterol synthesis in the crab *Cancer pagurus*. *Comp. Biochem. Physiol.*, **17**: 715–718.

VANDERZANT, E. S., 1963. Nutrition of the boll weevil larva. *J. Econ. Entomol.*, **56**: 357–362.

VANDERZANT, E. S., D. KERUR, AND R. REISER, 1957. The role of dietary fatty acids in the development of the pink bollworm. *J. Econ. Entomol.*, **50**: 606–608.

VIEHOEVER, A., AND J. COHEN, 1938. The response of *Daphnia magna* to vitamin E. *Am. J. Pharm. Educ.*, **110**: 297–315.

WICKSTEAD, J. H., 1965. *An introduction to the study of trophical plankton.* Hutchinson and Co., London, 160 pp.

ZANDEE, D. I., 1966. Metabolism in the crayfish *Astacus astacus.* III. Absence of cholesterol synthesis. *Arch. Int. Physiol. Biochim.*, **74**: 435–441.

ZANDEE, K. I., 1967. Absence of cholesterol synthesis as contrasted with the presence of fatty acids synthesis in some arthropods. *Comp. Biochem. Physiol.*, **20**: 811–822.

ZIMMERMAN, W. F., AND T. H. GOLDSMITH, 1971. Photosensitivity of circadian rhythms and of visual receptors in carotinoid-depleted *Drosophila. Science,* **171**: 1167–1169.

Reference: *Biol. Bull.*, **152**: 351–359. (June, 1977)

HISTOLOGY AND HISTOCHEMISTRY OF THE OVARY AND OOGENESIS IN *BALANUS AMPHITRITE* L. AND *B. EBURNEUS* GOULD (CIRRIPEDIA, CRUSTACEA)

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The oogenesis and microanatomy of the cirriped ovary are only partly described in earlier literature (Darwin, 1851, 1854; Krohn, 1859; Gruvel, 1905). More recently, ovarian maturation has been studied by visual observation of size, color and texture of the ovary mass (Crisp, 1954; Crisp and Davies, 1955; Patel and Crisp, 1960; Barnes, 1963; Barnes and Barnes, 1967) and by measurements of egg size (Crisp, 1954; Barnes and Stones, 1973). Utinomi (1960) and Turquier (1972) described briefly the ovary structure and oogenesis in acrothoracic cirripeds, and a brief description was made of cultured and uncultured ovarian tissue of the balanids (*Balanus amphitrite* and *B. eburneus*) by Fyhn and Costlow (1975). However, a more detailed microscopical examination of cirriped ovaries based upon histochemical techniques has not been available. The aim of the present study is to give data on the histology and histochemistry of the ovary and oogenesis of cirripeds represented by the two thoracic barnacles (*Balanus amphitrite* L. and *B. eburneus* Gould).

MATERIALS AND METHODS

Specimens of the acorn barnacle *Balanus amphitrite* (basal diameter 10 to 15 mm) were collected in October at the dock of Duke University Marine Laboratory, Beaufort, North Carolina. Specimens of *B. eburneus* (basal diameter 15 to 25 mm) were collected at the same place in August. The animals were fixed immediately upon collection. In addition, specimens of *B. amphitrite* collected during the months of April to June were fixed after being maintained for 10 to 14 days without food in aquaria at a salinity of 30‰ and 23 ± 1° C. The water was changed weekly, and the animals were then cleaned by light brushing. Prior to fixation some of the animals were molt staged according to the method of Davis, Fyhn and Fyhn (1973) using the rami of the sixth pair of cirri. The body and opercular valves were removed from the shell after cutting the opercular membrane. The shell with mantle tissue and ovarioles was fixed for two hours in acetic alcohol (3 parts of ethanol: 1 part of acetic acid). After washing in 100% ethanol, the mantle tissue was dissected out, embedded in Paraplast (Fisher Scientific) and serial sectioned at 8 μ. For general orientation, the Mallory-Heidenhain Azan stain was used (Koneff, 1938). For histochemical studies the following methods were used: (for proteins) Mercury-bromphenol blue after Bonhag (Pearse, 1968), Million reaction Baker modification (Pearse, 1968), Thioglycollate-ferric ferricyanide (Adams, 1956), DDD reaction (Barnett and

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Seligman, 1952) with alkylation for control, Thioglycollate/DDD reaction (Barnett and Seligman, 1952); (for carbohydrates) Toluidine blue O (Pearse, 1968; Lillie, 1929) with RNAse treatment for control, Azure A (Kramer and Windrum, 1955) with RNAse treatment for control, Mucihematein (Laskey, 1950) with RNAse treatment for control, Alcian blue (Steedman, 1950) with RNAse treatment for control, Periodic acid Schiff's reaction with acetylation and deacetylation for control (Casselman, 1962; Lillie, 1965), for DNA—Feulgen reaction (Feulgen and Rossenbeck, 1924), for DNA and RNA—Methyl-green-pyronin Y (Kurnick, 1955) with RNAse treatment for control; and (for lipids) Sudan black B (Pearse, 1968) with pyridine extraction for control.

Photomicroscopy was made with a Zeiss photomicroscope II and the size measurements were made with a *camera lucida* (magnification up to 813×).

RESULTS

The ovaries of *B. amphitrite* and *B. eburneus* consist of branched tubules (ovarioles) located in the connective tissue between the mantle cavity and the basal membrane. Four types of cells can be distinguished in the ovarioles (Fig. 1): cells of the ovariole membrane, oogonia, previtellogenetic oocytes in various stages of development (immature oocytes), and oocytes with discernible yolk droplets (mature oocytes). Interstitial or follicle cells were not observed around the oocytes. The cells of the ovariole membrane make up a continuous lining (ca. 0.25 to 0.50 μ thick) of the ovarioles. The cells are flattened with a cytoplasm staining light blue with Azan and showing weakly positive reactions in the tests for RNA and negative reactions in the other histochemical tests applied. Mitotic configurations were not observed in these cells. The oogonia are closely

TABLE I

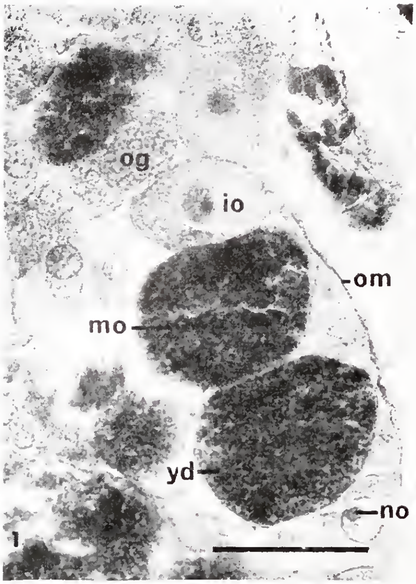
Histochemical reactions of the cytoplasm of immature oocytes and of yolk droplets of mature oocytes in Balanus amphitrite *and* B. eburneus.

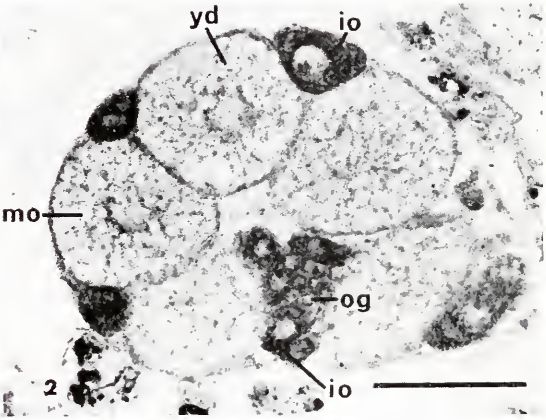
Test	Immature oocytes	Yolk droplets
Azan	yellow	red
Mercury-bromphenol blue	−	+++
Million reaction	−	+
Thioglycollate ferric ferricyanide	−	+++
DDD/control	−/−	++/−
Thioglycollic reduction, DDD	−	+++
Toluidine blue O/control	dark blue/−	−/−
Azure A/control	dark blue/−	−/−
Mucihematein/control	+/−	−/−
Alcian blue/control	bluegreen/−	−/−
Periodic acid Schiff's/control	++/−	+/−
Feulgen reaction	−	−
Methyl-green-pyronin Y/control	+++/−	−/−
Sudan black B/control	+/−	−/−

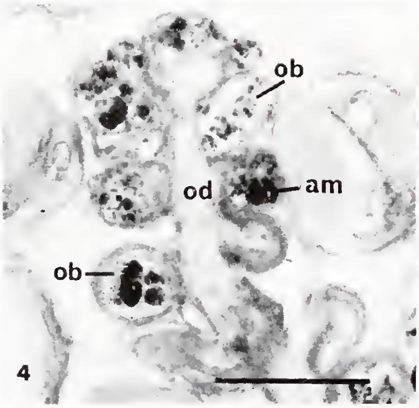
+++ Strongly positive reaction.
++ Moderately positive reaction.
+ Weakly positive reaction.
− Negative reaction.

packed cells making up a continuous string of cells in the ovariole. The cells are rounded with a diameter of about 2 μ and were frequently seen in mitosis. Pycnotic nuclei were not apparent in the oogonia. The cytoplasm of the oogonia showed positive reactions in the tests for RNA (Fig. 2) and negative reactions for carbohydrates and proteins. Immature oocytes (5 to 45 μ) have a large nucleus with one nucleolus (Fig. 1). Chromosome structures or premeiotic configurations (Raven, 1961) were not clearly seen. The histochemical reactions of the cytoplasm of immature oocytes are listed in Table I. The cytoplasm showed negative reactions in the tests for proteins (Fig. 3) and strongly positive reactions for RNA (methyl-green-pyronin Y, Fig. 2). The basophilic reactions observed by Toluidine blue O, Azure A and Alcian blue were absent after treatment with RNAse, indicating that the reactions with these stains might be due to RNA. No true metachromasia was observed. In the largest immature oocytes (diameter larger than 40 μ), the cytoplasm was more coarsely granulated and showed weaker basophilia than that of smaller oocytes. The cytoplasm showed a moderately positive reaction in the PAS test, which disappeared by acetylation and reappeared by deacetylation with KOH. This indicates that the positive reaction might be caused by 1, 2 glycol groups of carbohydrates. The cytoplasm showed a positive reaction with Sudan black B, and negative reaction after treatment with pyridine. The test was applied to fixed material, however, and gives positive reaction for large amounts of sudanophilic lipids only, since most of the lipids are dissolved by the fixative. Mature oocytes are approximately spherical with a diameter of 45 to 70 μ. The nucleus (10 to 12 μ) has one nucleolus. Meiotic chromosomes were not observed. The cytoplasm is filled with spherical yolk droplets with a diameter of 3 to 5 μ in *B. amphitrite* (Fig. 1) and 4 to 6 μ in *B. eburneus* (Fig. 3). In most oocytes the droplets were evenly distributed throughout the cytoplasm. In some oocytes, however, the periphery of the cytoplasm was free of droplets and the droplets were significantly smaller (1 to 3 μ in *B. amphitrite*) and less clear in appearance. Histochemical reactions of the yolk droplets of mature oocytes are shown in Table I. The droplets showed positive reactions in the protein tests (Fig. 3) and negative reactions in the tests for RNA (Fig. 2), acid mucopolysaccharides and mucin. The droplets showed weakly positive reactions for 1,2 glycol groups. The cytoplasm between the yolk droplets showed positive reactions in the tests for RNA (Fig. 2), lipids, and 1,2 glycol groups, and negative reactions in the tests for proteins (Fig. 3).

The ovarioles showed changes in structure and cellular composition. Small ovariole buds with a diameter of 20 to 25 μ in *B. amphitrite* (Fig. 7) and 20 to 30 μ in *B. eburneus* (Fig. 4) occurred along ovarian ducts in most of the animals. The ovariole membrane in the bud has a thickness of 1 to 1.2 μ in *B. amphitrite* and ca. 1.5 to 2 μ in *B. eburneus*. A high density of cell nuclei is observed in the membrane. In the lumen of the buds some cells can be seen, as well as amorphous material (Fig. 4). The latter is dark brown to black regardless of the staining technique applied. The ovariole buds seem to increase in length and width, the ovariole membrane becomes thinner, and its cell nuclei become more dispersed. No mitoses were observed in these nuclei. In slightly elongated ovarioles, a layer of columnar cells with apical nuclei has been organized beneath the ovariole membrane (Fig. 5). In more elongated ovarioles oogonia are found in the center







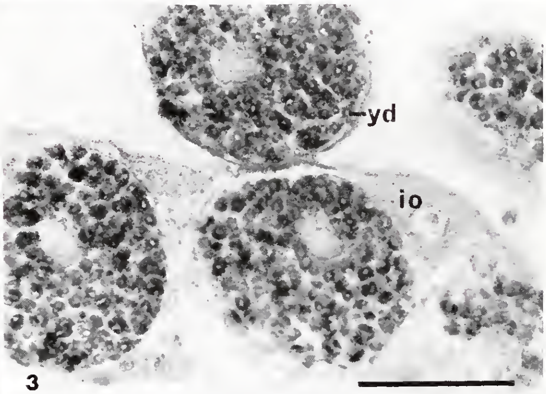


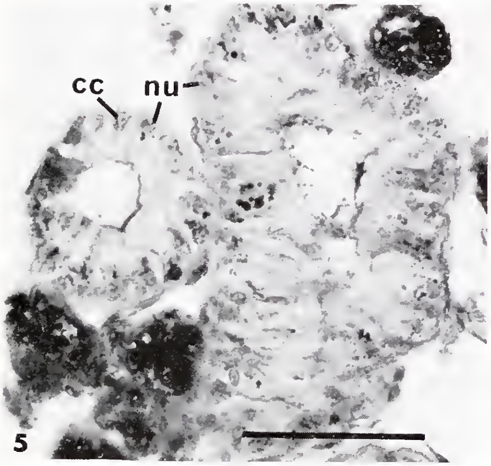
FIGURE 1. Ovariole of *B. amphitrite* with oogonia (og), immature oocytes (io) with nucleolus (no), mature oocytes (mo) with yolk droplets (yd) and ovariole membrane (om) (Azan stain, scale 50 μ).

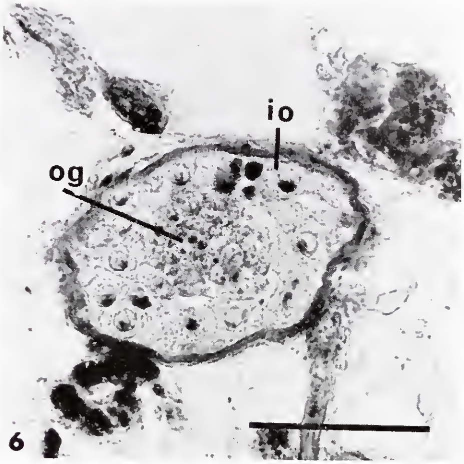
FIGURE 2. Ovariole of *B. amphitrite* with RNA in the cytoplasm of oogonia (og), immature oocytes (io), and between the yolk droplets (yd) in mature oocytes (mo) (methyl-green pyronin Y, scale 50 μ).

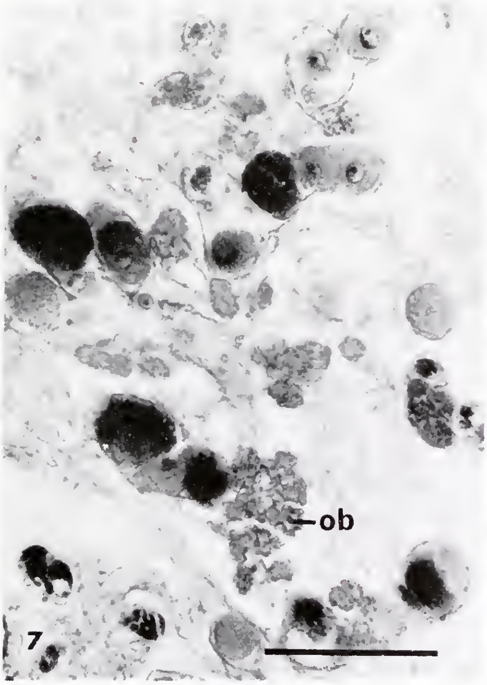
FIGURE 3. Ovariole of *B. eburneus* showing SH- and SS-positive yolk droplets (yd) and negative cytoplasm of immaure oocytes (io) (thioglycollic reduction followed by DDD reaction, scale 50 μ).

FIGURE 4. Ovariole buds (ob) with amorphous material (am) of *B. eburneus* along an ovarian duct (od) (Azan stain, scale 50 μ).

of the ovariole which has become a compact structure. The oogonia are observed in frequent mitoses. In even more elongated ovarioles, small immature oocytes are present in the periphery with the string of oogonia in the center (Fig. 6). As







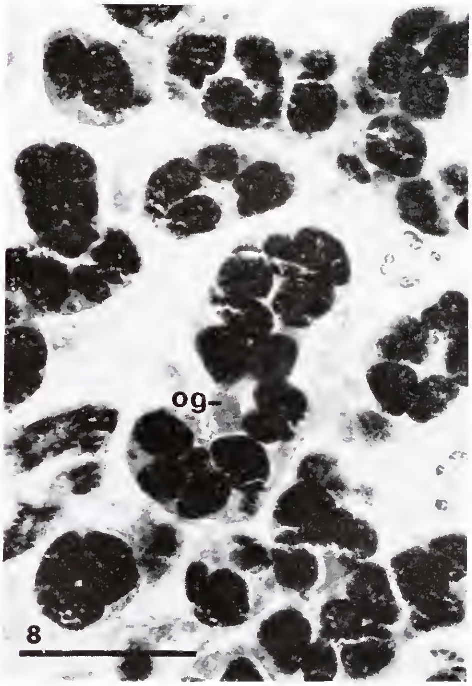


FIGURE 5. Ovarioles of *B. eburneus* with columnar cell (cc) having apical nuclei (nu) (Toluidine blue O, scale 50 μ).

FIGURE 6. Ovarioles of *B. eburneus* with oogonia in the center (og) and immature oocytes (io) in the periphery (Azan stain, scale 50 μ).

FIGURE 7. Ovarioles of *B. amphitrite* with ovariole buds (ob) and oocytes in various stages of development (Azan stain, scale 150 μ).

FIGURE 8. Elongated ovarioles of *B. amphitrite* with oogonia (og) and oocytes in various stages of development (Azan stain, scale 50 μ).

TABLE II

Ratio of mature to immature oocytes (total number of oocytes in parenthesis) and diameters (mean ± s.e. of the ten largest oocytes measured in each animal) of mature oocytes in ovarioles of Balanus amphitrite *in various stages of the intermolt cycle.*

Molt stage	Ratio of mature to immature oocytes ($>20\ \mu$)	Diameter of mature oocytes (μ)
B_1	0.35 (70)	48 ± 1.9
B_2 (early)	0.32 (111)	48 ± 1.1
B_2 (late)	0.76 (187)	55 ± 0.7
C	0.61 (135)	50 ± 1.6
D_1	0.85 (240)	68 ± 0.9
D_1	0.89 (66)	79 ± 1.5
D_2	0.74 (184)	58 ± 0.8
D_2	1.16 (261)	68 ± 2.0

the ovariole becomes further elongated, larger immature oocytes (more than 40 μ in diameter) and mature oocytes appear (Fig. 7). The number of mature oocytes increases with additional elongation of the ovariole (Fig. 8). The ovariole membrane is now 0.3 to 0.5 μ in thickness. The ovarioles are at this stage too long and undulated to be individually traced in serial sections. In transverse section the string of oogonia does not have a different location in the ovariole. Mitoses may be seen in the oogonia, and small immature oocytes (less than 20 μ) are located adjacent to the oogonia (Fig. 2). Immature oocytes of 20 to 30 μ are common in these ovarioles. There are no indications as to a nurse cell-function of these cells, and immature or mature oocytes did not appear in clusters.

Ovariole development and oogenesis were studied in relation to the intermolt cycle in eight specimens of *B. amphitrite* (molt stage B_1, B_2, C, D_1, and D_2) fixed immediately upon collection. Ovariole buds as well as elongated ovarioles with oogonia in mitoses, immature oocytes and mature oocytes were found in all stages of the intermolt cycle. The ratio of mature oocytes to immature oocytes larger than 20 μ in diameter was measured in sections stained with Toluidine blue O. This ratio increased from post- to proecdysial stages (Table II). The diameter (*i.e.,* the average of the longest and shortest diameter) of 20 to 50 mature oocytes of each animal was measured in sections stained with Azan. The mean of the ten largest oocytes in each animal showed a significant increase from postecdysis to proecdysis (Table II).

In animals maintained without food in aquaria for 10 to 40 days before fixation, immature oocytes larger than 20 μ in diameter were degenerating, and the degeneration became more evident with increasing time in aquaria. Smaller oocytes and oogonia seemed normal in most animals. The diameters of the mature oocytes were not significantly altered from the diameters in animals fixed immediately upon collection. The size and number of yolk droplets were normal.

DISCUSSION

A clear distinction between a germarium and a vitellarium was not found in the ovaries of *B. amphitrite* and *B. eburneus.* The germarium has no strictly defined localization but may be found in the periphery as well as in the center of the ovariole. The vitellarium consists of oocytes only, with apparently no accessory cells. Gruvel (1905) and Krüger (1940) claim that most of the oocytes in the ovary of pedunculate barnacles degenerate and function as a nutritional source for the growing oocytes. In branchiopods and ostracods, nurse cells are associated with the growing oocytes (Raven, 1961); and in decapods, the oocytes are surrounded by follicle cells (Herrick, 1911; Beams and Kessel, 1963). In anostracans (Linder, 1959) and isopods (Balesdent, 1965), the cells of the ovariole membrane have been described as follicular. In the present study of the barnacle ovary, no indications as to a nurse-cell function or a degeneration of immature oocytes were found. Follicle cells around the oocytes were not observed, and there was no evidence of a follicular function of the cells of the ovariole membrane. Most likely, therefore, the barnacle oocyte takes up nutrients through the cell membrane directly from its surroundings complying with the definition of an autonomous egg formation (Nørrevang, 1968).

The histochemical reactions of the developing oocytes and other ovarian cells in *B. amphitrite* and *B. eburneus* are mostly in agreement with descriptions of other crustaceans (Raven, 1961; Linder, 1959; Fautrez-Firlefijn, 1957). The formation of discernible proteid yolk droplets in the oocytes may be a rapid process, since oocytes containing only a few droplets were never observed. The periphery of the oocytes seems to be the last part to acquire yolk droplets. This is consistent with the finding in the acrothoracic cirriped *Trypetesa* that yolk droplets first appear in the perinuclear zone (Turquier, 1972). The sudden appearance of the yolk droplets in the barnacle oocytes does not necessarily reflect an equally rapid synthesis of yolk substances. The formation of yolk droplets may take place by a condensation process when yolk substances dispersed in the cytoplasm have reached a certain density.

The development of the ovary mass in cirripeds has macroscopically been shown to depend upon the food supply of the animal (Patel and Crisp, 1960; Crisp and Davies, 1955; Barnes and Barnes, 1967). In the present study, barnacles with well-developed ovaries were maintained for 10 to 40 days without food. During this starvation, the proteid yolk was maintained, while large and medium sized immature oocytes degenerated. This agrees with findings on barnacle ovary tissue maintained *in vitro* where proteid yolk was resistant to degeneration during insufficient nutritional supply, while previtellogenetic development was not maintained (Fyhn and Costlow, 1975).

During the breeding season, *B. amphitrite* has subsequent broods, one following closely after the other. In such species although the fertilization may take place at any time during an intermolt cycle, it most frequently occurs soon after ecdysis (Patel and Crisp, 1961). In the present study, mitotic activity in the oogonia and small oocytes adjacent to the oogonia was found in *B. amphitrite* of various stages of the intermolt cycle. This should indicate a continuous production of new oocytes throughout the intermolt cycle. However, an increase in the ratio of mature to immature oocytes from postecdysis to proecdysis was found, and the maximum size of mature oocytes showed an increase during the intermolt cycle. This may imply that vitellogenesis is initiated predominantly in postecdysis and is completed during one intermolt cycle.

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SUMMARY

Four cell types can be distinguished in the ovarioles of *Balanus amphitrite* and *B. eburneus:* cells in the ovariole membrane, oogonia, previtellogenetic oocytes in various stages of development, and oocytes filled with proteid yolk droplets. The germarium does not have a strictly defined localization in the ovariole. The vitellarium consists of oocytes only, with apparently no accessory cells. An autonomous egg formation seems probable. The histochemistry of the ovarian cells is described. The formation of discernible proteid yolk droplets appears to be a rapid process. The development from ovariole buds to elongated ovarioles is described. There is a continuous production of new oocytes throughout the intermolt cycle. Vitellogenesis seems to be initiated predominantly in postecydsis and is completed during one intermolt cycle.

LITERATURE CITED

ADAMS, C. W. M., 1956. A stricter interpretation of the ferric ferricyanide reaction with particular reference to the demonstration of protein-bound sulphydryl and di-sulphide groups. *J. Histochem. Cytochem.,* **4**: 23–35.

BALESDENT, M. L., 1965. Recherches sur la sexualité et le déterminisme des caractères sexuales d'*Asellus aquaticus* Linné (Crustacé Isopode). *Bull. Acad. Soc. Lorraines Sci.,* **5**: 1–231.

BARNES, B., 1963. Light, temperature and the breeding of *Balanus balanoides. J. Mar. Biol. Assoc. U.K.,* **43**: 717–727.

BARNES, H., AND M. BARNES 1967. The effect of starvation and feeding on the time of production of egg masses in the boreo-arctic cirripede, *Balanus balanoides* (L.). *J. Exp. Mar. Biol. Ecol.,* **1**: 1–6.

BARNES, H., AND R. L. STONES, 1973. The general biology of *Verruca stroemia* (O. F. Müller). II. Reproductive cycle, population structure, and factors affecting release of nauplii. *J. Exp. Mar. Biol. Ecol.,* **12**: 279–297.

BARNETT, R. J., AND A. M. SELIGMAN, 1952. Histochemical demonstration of protein-bound sulfydryl groups. *Science,* **116**: 323–327.

BEAMS, H. W., AND R. G. KESSEL, 1963. Electron microscope studies on developing crayfish oocytes with special reference to the origin of yolk. *J. Cell Biol.,* **18**: 621–649.

CASSELMAN, W. G. B., 1962. *Histochemical techniques.* Methuen and Co. Ltd., London, 205 pp.

CRISP, D. J., 1954. The breeding of *Balanus porcatus* (da Costa) in the Irish Sea. *J. Mar. Biol. Assoc. U.K.,* **33**: 473–494.

CRISP, D. J., AND P. A. DAVIES, 1955. Observations *in vivo* on the breeding of *Elminius modestus* grown on glass slides. *J. Mar. Biol. Assoc. U.K.,* **34**: 357–380.

DARWIN, C., 1851. *A monograph on the sub-class Cirripedia. The Lepadidae.* Ray Society, London, 400 pp.

DARWIN, C., 1854. *A monograph on the sub-class Cirripedia. The Balanidae.* Ray Society, London, 684 pp.

DAVIS, C. W., U. E. H. FYHN, AND H. J. FYHN, 1973. The intermolt cycle of cirripeds: criteria for its stages and its duration in *Balanus amphitrite. Biol. Bull.,* **145**: 310–322.

FAUTREZ-FIRLEFIJN, N., 1957. Protéines, lipides et glucides dans l'oeuf d'*Artemia salina. Arch. Biol.,* **68**: 249–296.

FEULGEN, R., AND H. ROSSENBECK, 1924. Mikroskopisch-chemischer Nachweis einer Nucleinsaure vom Typus der Thymonucleinsäure und die darauf beruhende elektive Farbung von Zellkernen in mikroskopische Präparaten. *Hoppe-Seyler's Z. Physiol. Chem.* **135**: 203–248.

Fyhn, U. E. H., and J. D. Costlow, 1975. Tissue cultures of cirripeds. *Biol. Bull.,* **149**: 316–330.

Gruvel, A., 1905. *Monographie des Cirripedes ou Thécostracés.* Masson and Co., Paris, 472 pp.

Herrick, F. H., 1911. Natural history of the American lobster. *Bull. U. S. Bur. Fish.,* **29**: 149–408.

Koneff, A. A., 1938. Adaption of the Mallory-Azan staining method to the anterior pituitary of the rat. *Stain Technol.,* **13**: 49–52.

Kramer, H., and G. M. Windrum, 1955. The metachromatic staining reaction. *J. Histochem. Cytochem.,* **3**: 227–237.

Krohn, A., 1859. Beobachtungen über das Cementapparat und die Weiblichen Zeugungsapparate einiger Cirripedien. *Wiegmann's Arch. Naturgesch. Berlin,* **25**: 355–364.

Krüger, P., 1940. *Cirripedia. Bronn's Klassen und Ordnungen des Tierreiches.* Bd. 5, Abt. 1, Buch 3, Teil 3. Academische Verlagsgesellschaft, Leipzig, 297–342 pp.

Kurnick, N. B., 1955. Pyronin Y in the methyl green-pyronin histological stain. *Stain Technol.,* **30**: 213–230.

Laskey, A. M., 1950. A modification of Mayer's mucihematein technic. *Stain Technol.,* **25**: 33–34.

Lillie, R. D., 1929. A brief method for the demonstration of mucin. *J. Tech. Meth. Bull. Int. Assoc. Med. Mus.,* **12**: 120–121.

Lillie, R. D., 1965. *Histopathologic technic and practical histochemistry,* 3rd ed. McGraw-Hill, Inc., New York, 715 pp.

Linder, H. J., 1959. Studies on the fresh water fairy shrimp *Chirocephalopsis bundyi* (Forbes) I. Structure and histochemistry of the ovary and assessory reproductive tissues. *J. Morphol.,* **104**: 1–60.

Nørrevang, A., 1968. Electron microscopic morphology of oogenesis. *Int. Rev. Cytol.,* **23**: 113–186.

Patel, B., and D. J. Crisp, 1960. The influence of temperature on the breeding and moulting activities of some warm-water species of operculate barnacles. *J. Mar. Biol. Assoc. U.K.,* **39**: 667–680.

Patel, B., and D. J. Crisp, 1961. Relation between the breeding and moulting cycles in cirripedes. *Crustaceana,* **2**: 89–107.

Pearse, A. G. E., 1968. *Histochemistry: theoretical and applied,* 3rd. ed., Vol. I. Little, Brown and Co., Boston, 759 pp.

Raven, C. P., 1961. *Oogenesis: the storage of developmental information.* Pergamon Press, New York, 274 pp.

Steedman, H. F., 1950. Alcian blue 8GS: a new stain for mucin. *Q. J. Microsc. Sci.,* **91**: 477–479.

Turquier, Y., 1972. Recherches sur la biologie des Cirripèdes Acrothoraciques VI. Contribution à l'étude de la reproduction et du déterminisme du sexe chez les Trypetesidae des côtes françaises. *Arch. Zool. Exp. Gen.,* **113**: 167–196.

Utinomi, H., 1960. Studies on the Cirripedia Acrothoracica II. Internal anatomy of the female of *Berndtia purpurea* Utinomi. *Publ. Seto Mar. Biol. Lab.,* **8**: 223–279.

Reference: *Biol. Bull.*, **152**: 360–372. (June, 1977)

A GASTROPOD COLOR POLYMORPHISM: ONE ADAPTIVE STRATEGY OF PHENOTYPIC VARIATION

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Most species of the genus *Crepidula* possess very few discrete, multistate traits, but rather show subtle gradations in characters. There are actually very few characters in which polymorphism can be observed, because ornamentation is lacking in most species, and shell shape is a plastic character, strongly affected by the shape of the substrate. However, *Crepidula convexa* does show distinct, nonintergrading color differences within single populations.

The external colors of many species of *Crepidula* are often obscured by algal growth, as well as by a shaggy periostracum. *C. convexa*, however, has a thin periostracum of the same hue as the shell. Being small and able to move, at least in the male phase, it does not become encrusted by advancing colonies of epibionts (bryozoans, algae, tunicates) as do other species. It is photopositive and prefers the upper surfaces of substrates (personal observation), hence its color is visible to other organisms.

The limpet-like *C. convexa* requires smooth, hard substrates in intertidal to shallow subtidal waters. Such substrates—especially the inner surfaces of other shells, polished slate or quartz stones, glass, or, if nothing else exists, eel grass blades—exist in spatially heterogeneous patches. They are also impermanent in time but not usually within the generation time of individual *C. convexa*.

The most common color of the shell of this northern Atlantic species is purplish-grey, with a rich, dark brown interior. Darker rays of pigment extend radially from the apex but are visible only at the shell margin where the background color is less intense. A small proportion of pale tan or yellow shells, with reddish-brown rays, coexists with the darker specimens. An even smaller number of light brown specimens with uneven pigmentation (streaks of yellow mixing with the brown) is usually present. Franz and Hendler (1970) noted these color differences, along with the fact that there were more pale shells living on cultch (clam shell debris) than on gastropod shells. Color differences are discrete, with scoring relatively uncomplicated by ambiguous intermediates.

Color polymorphism in *C. convexa* is of interest because it is a rare occurrence in the genus. Analyses of shell color, substrate color, and relative fitness of individuals of each color on each substrate were performed to investigate how such a polymorphism is maintained in this species. *C. convexa* was compared with one other species which shows discrete color classes, and with species lacking color polymorphism to investigate why it exists in this but not most other species of *Crepidula*.

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MATERIALS AND METHODS

The color, sex, and size (length and height, in mm) of each specimen found in two populations of *C. convexa* were recorded as part of a sampling program during the summer of 1973. The type and color of the substrate on which each snail rested were also recorded. Colors were observed while specimens and substrates were wet. One population was located in the vicinity of the Woods Hole Yacht Club near Penzance Point, Woods Hole, Massachusetts. The other was in Waquoit Bay, Massachusetts. Both populations extended from the lower intertidal zone to three feet below the low tide line. The substrates consisted of small stones, clam and other mollusc shells, scattered eel grass, and trash (glass, porcelain, and cans) on a sandy bottom in the case of the Woods Hole locality, and a mud bottom at Waquoit Bay.

The data were analyzed by means of Chi-square tests to see if shell color correlated with substrate color, or with size or sex. An analysis of the polymorphism in *C. convexa* in light of the fitness-set theory developed by Levins (1968) and applied by McNaughton (1970) and Childress (1972) was conducted. This involved finding a measure of fitness and plotting it for a series of phenotypes (colors) on one substrate *versus* fitness of the same phenotypes on a second substrate. This plot is called a fitness set. Finally, motility of adults and juveniles, sources of predation, and substrates available within the geographical range of the populations studied were observed and recorded for *C. convexa*. The shape of the fitness set was then evaluated in the context of these several ecological factors to

TABLE I

Color polymorphism in Crepidula convexa.

Population	Substrate	Color	Color of *C. convexa*			Total
			Purple	Brown	Tan	
Woods Hole July, 1973	*Littorina*	brown to purple	108	2	6	116
	Porcelain	white to tan	64	2	10	76
	Total numbers:		172	4	16	192
	Per cent:		89.6%	2.1%	8.3%	100%
Woods Hole Sept., 1973	*Littorina*	brown to purple	75	7	7	89
	Glass	brown or green	104	6	4	114
	Porcelain	white	8	1	4	13
	Clam shells	white or greyish	9	1	2	12
	Total numbers:		196	15	17	228
	Per cent:		85.9%	6.6%	7.5%	100%
Waquoit Bay Sept., 1973	Eel grass	covered with tan epiphyte	32	3	16	51
	Nassarius	dark purple	9	0	1	10
	Littorina	brown to purple	33	1	3	37
	Clam shells	white or grey	35	3	11	49
	Stones	white (quartz), some dark	49	2	13	64
	C. fornicata	reddish brown	25	5	6	36
	Total numbers:		183	14	50	247
	Per cent:		74.1%	5.6%	20.2%	100%

determine whether or not the observed color polymorphism could be stable in an evolutionary sense.

RESULTS

Tables I and II summarize the raw data. No significant correlation between the intensity categories of pigmentation and size categories of the specimens was found, using a Chi-square test of independence ($0.10 < P < 0.25$). Thus, intensity of pigmentation is not a function of age. The largest five individuals, three purple and two tan, were found on white substrates. This is probably a consequence of *C. convexa* growing to larger dimensions on flat surfaces, which coincidentally tend to be of light shade in the localities studied. Large specimens (the females) were better matched to their substrates in intensity of pigmentation than juveniles (Table II). Individuals were uniform in color.

Table I clearly shows that there are far fewer of the intermediate class (brown color) than predicted by the Hardy-Weinberg ratio, under one possible hypothesis—the simplest and most convenient assumption in lieu of breeding tests—that the color system is controlled at one locus by two alleles, with the intermediate color equivalent to the heterozygous class. This assumption is supported by the discreteness of the color morphs.

Table III shows that the color of the shell is not independent of the color of the substrate. There is a higher percentage of tan animals on light backgrounds and of dark animals on dark backgrounds than expected by random distribution. In both the Waquoit Bay and the Woods Hole populations, the association of shell shade with substrate shade deviates significantly from randonmess at the 0.005 level. The overall percentage of tan *versus* purple shells differs in the two populations but is constant in two samplings of the same population at a two-month interval (Table I). There are proportionally more tan shells in the Waquoit population, which has the greater percentage of light substrates (about 60% of the exposed surface area in randomly sampled quadrats) than in the Woods Hole population, where 75% of the available substrate is dark. The percentages of intermediate-colored substrates at both Woods Hole and Waquoit Bay are 10%; at Woods Hole these substrates are virtually unoccupied by *C. convexa*. The densities of

TABLE II

Color matching of C. convexa *and substrate by size and sex (J = juvenile; M = male; I = intermediate, changing sex from male to female; F = female). Individuals of the Waquoit Bay and Woods Hole populations have been pooled for this analysis. A match consists of a light snail on a light background, or a dark snail on a dark background.*

Sex	L(mm)	Matched	Mismatched	Percentage matched
J	>0–3	82	90	0.48
M	>3–6	121	51	0.70
M, I, F	>6–9	63	41	0.61
F	>9–12	118	45	0.72
F	>12–15	40	16	0.71
Total		424	243	0.64

Table III

Independence test of the color of the snail shell versus shade of the substrate (O = number observed; E = number expected under the null hypothesis that the color of the snail shell is independent of the color of the substrate.

A. Waquoit population ($\chi^2 = 34.2$; $P < 0.005$)

Substrate (Percentage of surface area)	Snail shell color									Total
	Purple			Brown			Tan			
	O	E	χ^2	O	E	χ^2	O	E	χ^2	
Light (60%)	77	93	2.8	8	7	0.1	40	25	9.0	125
Neutral (10%)	25	27	0.2	5	2	4.5	6	7	0.1	36
Dark (30%)	81	64	4.5	1	5	3.2	4	17	9.9	86
Total	183			14			50			247

B. Woods Hole population, two samples pooled ($\chi^2 = 11.8$; $P < 0.005$)

Substrate (Percentage of surface area)	Purple O	Purple E	Purple χ^2	Brown O	Brown E	Brown χ^2	Tan O	Tan E	Tan χ^2	Total
Light (15%)	81	89	0.7	4	5	0.2	16	8	8.0	101
Dark (75%)	287	280	0.2	15	14	0.1	17	25	2.6	319
Total	368			19			33			420

shells on dark and light substrates are similar, showing that *C. convexa* does not preferentially occupy either dark or light substrates. However, there are fewer shells on intermediate substrate shades.

Chi-square values of each substrate calculated independently (1 d.f.) are shown in Table III, in addition to the summed Chi-square value for all comparisons. Most of the individual results are significant, and in the case of the Waquoit population, reveal that the least significant values are for adjacent color combinations (purple shells on neutral substrate, brown shells on light substrate, and tan shells on neutral substrate) as opposed to dark shells on light substrates.

Despite the fact that the gene frequencies in the populations studied here may not be in equilibrium with the immediate environment and it is therefore not possible to conclude that the polymorphism observed is stable, the question of whether or not a balanced polymorphism *could* exist in *C. convexa* is worth pursuing. Application of the data to fitness sets (Levins, 1968) gives insights into why other species of *Crepidula* with different life history patterns should not favor polymorphism. But only if balanced polymorphism in *C. convexa* is assumed may the data be used as a true test of the model. Conversely, to test the possibility of balanced polymorphism, the validity of the model must not be in question. For the present, one can only say whether or not Levins' model is consistent with data on polymorphism in *C. convexa*. It is important that field data be applied to models such as Levins', to increase the dialogue between theoretical and field ecologists.

If the plot of the fitnesses of an ordered series of phenotypes in one environment (W_1) *versus* the fitnesses of the same phenotypes in another environment

(W_2) is concave in the region between the maximum fitness value in each of the two environments, and if the environments are coarse-grained with respect to the organism, then Levins predicts that polymorphism will be the optimal population strategy. Applying the theory to *C. convexa*, first an estimate of fitness is needed. Clutch size is one means, but is highly dependent on age and size of the female, since these snails continue to grow past maturity and clutch size is proportional to size of the female. The factor most important in individual reproductive success, and hence in estimating fitness of the genotype, is the life span of the individual. In the populations studied, the two "environments" were the dark and the light substrates. They did not differ in food availability to these filter-feeders, or in major physical factors such as temperature, so any differential in size was assumed to be a function of age, not nutrition. This was verified by observing that the distance between major growth lines representing winter growth cessation was similar in equal-sized snails from different substrates in the same populaton ($P > 0.5$).

Because fitness of the genotype of any individual based upon its life span cannot be obtained by instantaneous measurement, a modified estimator of fitness was used. This was the relative fitness for a group of animals, obtained by finding the mean size of females of each color on each substrate. This produced estimates of differential survival and hence differential reproductive success of the three color morphs of *C. convexa* on each of the substrate "environments." This method assumes a constant yearly recruitment rate and stable age distribution, which are borne out by field studies of recruitment as well as population age and size structure of *C. convexa* (Hoagland, 1975). Such assumptions are invalid for species of *Crepidula* with planktonic larvae, such as *C. fornicata*. Another important assumption is that once mature, females are sedentary and are henceforth subjected to selection pressures particular to the substrate on which they are found.

Table IV gives the results of this analysis, taking into consideration the mass as well as the length of the shell in the estimate of size. Length is a biased estimator of size because some of the dark substrates (*Nassarius* and *Littorina* shells, primarily) are highly convex surfaces, and snails living on them grow proportionally taller than do those on the light substrates (primary clam shells and pieces of porcelain). Records of length and dry weight for a series of 50 individuals from each habitat showed a 10% greater biomass in snails of a given length living on *Nassarius* and *Littorina*, relative to those living on stones and clams. Therefore the lengths of snails living on these substrates were cor-

TABLE IV

Differential survival of C. convexa *on two colors of substrate.*

C. convexa Shell pigmentation	Mean length in millimeters		
	Light substrate	Dark substrate	Dark substrate, corrected length
Purple	9.5 ± .2*	10.1	11.1 ± .3
Brown	9.8 ± .3	9.3	10.2 ± .3
Tan	11.5 ± .4	8.9	9.8 ± .2

* Mean ± standard error.

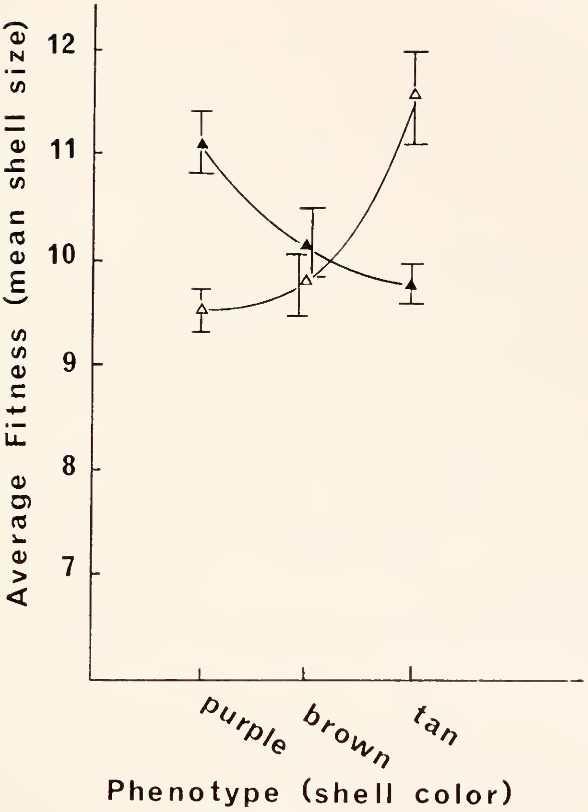

FIGURE 1. Average fitness as a function of phenotype of *Crepidula convexa* in two environments. Fitness is estimated as mean shell length for all female individuals of each color on each substrate (environment). Vertical bars are one standard error unit. Open triangles represent light substrates; and solid triangles, dark substrates.

rected by multiplying them by a factor of 1.1 (last column of Table IV). Figure 1 is a plot of the phenotype (color) *versus* average fitness (survival, as measured by shell size), for the two environments. Figure 2 is a plot of the resulting fitness set, for the critical region between W_1 (max) and W_2 (max). It shows that, indeed, the fitness set is concave. This is because the survival of the intermediate phenotype, the brown shells, is low on both dark and light substrates. In the terminology of this paper, it makes a "mismatch" on either substrate. The heavy mortality of this poorly-adapted heterozygote represents the cost of the strategy of polymorphism to *C. convexa;* it must be outweighed by the fitness of the two extreme color morphs in the heterogeneous environment in order for polymorphism to be a viable strategy. The sedentary females have higher survival on matched substrates.

General ecological observations complement the fitness set analysis. Records of species living in Vineyard Sound (Verrill and Smith, 1874) reveal that the intertidal to shallow-water populations of *C. convexa* are not isolated but are in

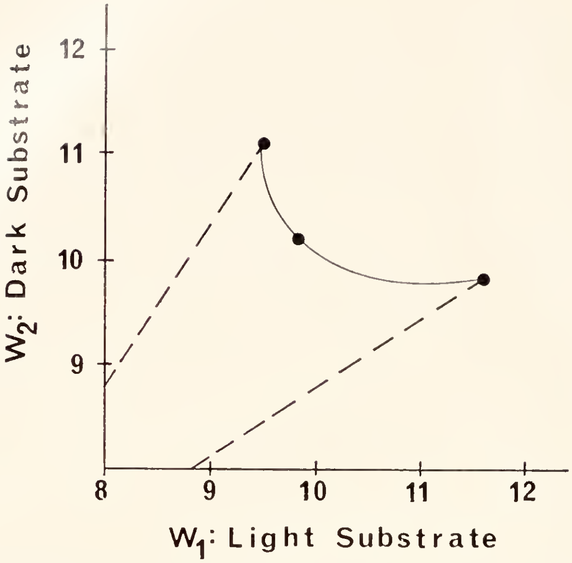

FIGURE 2. A fitness set for *Crepidula convexa* on two substrates. The points represent the three color morphs (purple, brown, and tan) from left to right. The X coordinates (W_1) are the mean lengths of each morph on light substrate; the Y coordinates (W_2) are the mean lengths of each morph on dark substrates.

communication with deeper-water populations and probably also those of other shallow water areas. The demes are separated by unsuitable substrate (sand), but individuals are transported by plant and animal hosts, as demonstrated by the discovery of young *C. convexa* on fragments of eel grass and especially on horseshoe crabs and hermit crabs which migrate to deeper waters in winter. Gene flow is probably high enough to maintain an unbalanced polymorphism without speciation, evidence for which is present in over one hunderd years of shell specimens from the New England area now housed in American museums (Academy of Natural Sciences, Philadelphia: Museum of Comparative Zoology).

One seemingly anomalous fact is that there are available to most populations of *C. convexa* not only black and white stones and shells, but grey stones as well, usually mottled granites. Juvenile individuals of *C. convexa* occasionally settle on these, but very few adults are ever found on them. Rocks of mixed or intermediate shades are usually of heterogeneous composition and are rough in texture, for they erode unevenly. Individuals of *C. convexa* avoid these in favor of smooth substrates, as seen by placing specimens of *C. convexa* in laboratory tanks with various substrates available to them. Thus, there is a dichotomy of substrate shades available to *C. convexa* in the sites studied, despite the presence of other shades in the environment.

It is important in evaluating the stability of the color polymorphism to know how free *C. convexa* is to move from one substrate to another. Motility of *C. convexa* was observed in the field and in laboratory tanks. Both males and females showed high motility during the first one to two days after placement of

50 individuals of each sex in a laboratory tank. If smooth substrates were available, females became stationary after the initial one to two day period. If tank conditions were altered to increase water temperature or to reduce the amount of oxygen in the water, *C. convexa* females became motile again, unless they were brooding young. Brooding females were completely sedentary. Males became stationary on females for periods ranging from a few hours to 29 days, most frequently 20 days. After such a stationary period, a male either separated from the female and changed sex, or found another female.

In the field, marked individuals of *C. convexa* were difficult to recover because their substrates were either living and moving themselves, or were moved by tides and currents. Young individuals (less than 5 mm long) of *C. convexa* appeared to be highly motile, but brooding females were sedentary and some males were associated with females for periods as long as two months.

To see if shell color could directly affect mortality of individual *C. convexa* and hence be strongly controlled by natural selection, possible causes of mortality were examined. No specimens of *C. convexa* were found which had been drilled by gastropod predators. Starfish were never seen attacking *C. convexa* or the *Nassarius* or *Littorina* on which they lived, though starfish were found on nearby mussel beds. Crabs were frequently observed eating *C. convexa* and young *Littorina littorea.* Once a gull was seen smashing the shell of a *L. littorea* which had two *C. convexa* attached to it. These observations indicate that visual predators which could detect pigment differences (crabs and birds) might be important in killing *C. convexa*, while primarily olfactory predators such as starfish and drilling gastropods are probably less important.

Discussion

Several assumptions have been used in interpreting the data so far presented. The most critical is that pigmentation is a genetic character basically unchanged by the environment, and constant throughout the lifetime of any individual. Moore (1936) suggested that diet plays a large role in the color variation of *Purpura* (= *Nucella*) *lapillus.* However, because all species of *Crepidula* are filter-feeders, their diets are less likely to be the cause of cryptic coloration than those in animals that feed directly on the substrate they occupy. The fact that no individuals of *C. convexa* were found which changed color in mid-growth is consistent with the assumption. Also, the assumption that a genetically controlled color system is controlled at one locus is compatible with the genetic bases of other molluscan color polymorphisms (Cain and Sheppard, 1950, 1954; see also Komai and Emura, 1955). However, the exact genetic mechanism is not critical to the argument developed below.

One might ask if the color morphs of *C. convexa* are really different species. The possibility is discounted by the presence of intermediates, male-female pairings between the morphs in the laboratory (such pairings do not occur between species), simultaneous breeding times, identical larvae, identical morphologies other than color, and by similar maximum size and lifespan.

Incipient speciation through geographical isolation is an unlikely explanation for discrete color morphs, but is hard to disprove without direct evidence on gene flow and gene frequencies over time. However, *C. convexa* is identifiable in the fossil record from the mid-Miocene to the present, with a range from Nova Scotia to Western Florida, without the development of species complexes of closely related forms such as occurs in *Littorina* (Borkowski and Borkowski, 1969).

Taking the body of fact and assumptions presented, it appears that selection is operating to reduce the numbers of the intermediate color type and to favor dark over light shells in *C. convexa* on dark backgrounds. Benefit also accrues to light shells on light backgrounds. Either the snails are able to find a substrate of matching color, or predation causes a reduction in the number of mismatched snails, or both. Because larger specimens of *C. convexa* were better matched to the background color than younger and smaller specimens, predation could be acting to weed out mismatched individuals. The lower percentage of matched snails at the length range of six to nine mm (Table II) is due to the intermediate sexual stage occuring at about that size. The animals change substrates at the time of their sex change to female, because they then move off their mates and appear to be repelled by them. They again must sort themselves out as to color background, possibly through a behavioral mechanism. The behavioral mechanism is unknown and its existence unproven. Such a mechanism was hypothesized by Giesel (1970) to explain the matching of *Acmaea digitalis* to its substrate, but that case is complicated by homing behavior in the adult limpets (Fretter and Graham, 1962, p. 498). *C. convexa* does not home.

Opposite proportions of dark and light substrate in neighboring localities could affect the gene frequencies by creating selection pressures favoring different colors of *C. convexa* in different places, with some mixing occurring due to the transportation of adults. Additionally, the subtidal portion of each *C. convexa* population may not be under the same intensity of visual predation as that portion which is intertidal. The behavioral trait of being able to match shell shade to substrate, if it exists, would have a stabilizing effect on the color polymorphism in any one population. It would be of interest to study an isolated population of *C. convexa* where gene flow to and from other populations is very low, to see if one or the other pigment shade became fixed, or if the polymorphism could be balanced.

The concave fitness set resulting from the plot of fitnesses of different shell colors on light and dark substrates is compatible with the hypothesis that color polymorphism in *C. convexa* is balanced. Levins' fitness set model for balanced polymorphism further requires that the environments be "coarse-grained" with respect to the organism in order for polymorphism to evolve. An organism is coarse-grained if it spends disproportionate time in one of several temporally or spatially arranged habitat patches within a heterogeneous environment (Gillespie, 1974; Maynard Smith, 1970; Levins, 1968; Levene, 1953). The limited mobility of *C. convexa,* combined with its small size relative to the habitat patches (rocks, shells, and sand), its brooded (nonplanktonic) young, and its hypothesized ability to choose a substrate befitting it, makes it coarse-grained with respect to the spatial environment. This completes the criteria necessary and sufficient for *C. convexa* to possess balanced color polymorphism. An individual *C. convexa* may spend a major portion of its lifetime in one of several contemporaneous habitat patches, while its offspring may spend their lifetimes in different patches.

Therefore, it is possible that *C. convexa* can maintain a color polymorphism without genetic exchange between populations. However, the proximity of other habitats with different proportions of substrate types and potential predators to the populations studied, as well as a means for individuals to migrate between the habitat areas, suggests the possibility of polymorphism based on genetic exchange within the Woods Hole area.

C. convexa is unable to accommodate ice scour, occurring every three to seven years in the cold temperate marine intertidal zone. This is due to the short lifespan of the individual and marks it as coarse-grained with respect to time. However, this factor is probably not relevant to color polymorphism, unless the severe weather were to coincide with substrate disruption, which does not happen in the localities studied. Temporal environmental fluctuations might be expected to cause polymorphism in metabolic characters.

Looking at other members of the genus *Crepidula*, *C. adunca* is the only one which shows color polymorphism. This Pacific species is ecologically similar to *C. convexa*. It is chocolate brown or yellow, with very little representation of intermediate shades. As with *C. convexa*, the question is one of intensity of pigmentation rather than presence of different colors. Populations in Monterey, California, contain a high proportion of dark shells, while some from Southern California are primarily yellow. Some populations are entirely one color or the other (personal observation). Besides predation, pigmentation of intertidal gastropod shells may be a factor in regulating heat absorption during exposure at low tide. Darker shells absorb more heat and may be disadvantaged in southern climates. Furthermore, the percentage of dark backgrounds in Monterey is over 50%, while it is less than 50% in most Southern and Baja California localities, suggesting that the predominant color of *C. adunca* may be due to camouflage.

A conflicting adaptive context would exist if a population had to cope with a hot environment, while the majority of substrates were dark. The strategy for camouflage (dark color) would conflict with the strategy for heat loss (light color). An interesting test of the importance of heat loss *versus* camouflage could be undertaken if specimens of *C. adunca* were found living on predominantly dark substrates in the southern part of its range. Examination of such a population would give insight into the dominant selective pressure for color polymorphism in this species.

Unique conditions relevant to color polymorphism shared by *C. convexa* and *C. adunca* are that their inherited shell color is usually not obscured by epibionts, they frequent the intertidal zone particularly by attaching themselves to the shells of intertidal gastropods, and they are subject to predation by gulls, shore birds, and crabs more consistently than by starfish and predatory gastropods, according to field observations and laboratory tests (Hoagland, 1974). All visual predators attack all size classes, probably because the range of size within these species is small (1–20 mm). Birds and crabs are visual predators, whereas starfish and gastropods such as *Urosalpinx* and *Thais* rely more on olfactory stimuli (Owen, 1966, pp. 23–25; Carriker, 1955). Strictly subtidal species of *Crepidula* do not exhibit color polymorphism (Hoagland, 1977). They are heavily preyed upon by olfactory predators such as starfish. However, the reason for the difference in types of predators is only partly the vertical position of the *Crepidula* on the shoreline. Size of the individuals and population density are important factors. Starfish and drilling gastropods have definite hierarchical prey preferences (Paine, 1969), and they virtually ignore the small and relatively rare *C. convexa* and *C. adunca* during the feeding periods of these predators. They prefer mussels, the larger and more numerous sympatric species of *Crepidula* (*C. onyx* in the Pacific and *C. fornicata* in the Atlantic), or barnacles, in the case of *Urosalpinx* in the intertidal zone at Woods Hole (Hoagland, 1974).

The small intertidal species of *Crepidula* lack a planktonic larval stage and live for only two to four years, compared with an estimated maximum life span of eight to ten years for the larger *Crepidula* species (Hoagland, 1975). Perception of environmental heterogeneity therefore must differ for individuals in the two groups of species. For example, *C. fornicata* has low mobility, but appears to be fine-grained in its choice of substrates. In the laboratory, it does not distinguish substrates on the basis of micro-texture. It settles on exposed and sheltered surfaces, horizontal and vertical. This is one of several characters by which it may be termed a generalist—temperature and salinity tolerance are others. The significance of the generalist mode is that it, along with the planktonic larval form, enables individuals of *C. fornicata* to excel in establishing themselves in new areas. Their longer life span relative to *C. convexa* means that adaptation to cyclical environmental heterogeneity is possible and desirable. If the range of the environment is less than the tolerance of individual phenotypes, a convex fitness set should result. One could predict, on the basis of grain size, low polymorphism. There is no evidence of visually detectible polymorphism in *C. fornicata* or any ecologically similar species (*C. onyx; C. dilatata*), but rather there is phenotypic plasticity, a strong molding of each individual by its immediate environment.

Following the lines of this argument, species with homogenous habitats are not expected to have either high polymorphism or plasticity. Species with low visual predation are unlikely to possess color polymorphism. *C. plana* and other photonegative *Crepidula* fall into this category. Their habitat preferences are narrowly defined, restricted to the under- or inner-surfaces of solid objects. *C. plana* is primarily subtidal, except when it lives on the undersides of living organisms where it is continually kept moist and is not readily visible to sight predators. Therefore, selective pressure for color matching with the substrate is not a significant factor in the biology of *C. plana*.

Balanced polymorphism is an alternative to phenotypic plasticity, in that both strategies allow a population, on a statistical basis, to broaden its niche (to extend over a diversity of substrates) when occupying a habitat which is unpredictably heterogeneous in space or time. In the genus *Crepidula,* expression of color polymorphism is related to spatial heterogeneity. Its presence depends upon the particular species' lifespan, mobility, substrate preferences, and mode of major predators' attack. In *C. convexa,* each of two color morphs is superior on a particular substrate, while intermediate forms are less successful.

Other polymorphic characters, such as enzymes, might be expected to be found in *C. convexa* and *C. adunca* populations as a result of temporal heterogeneity of their environments. Polymorphism is the favored strategy when, as with *C. convexa,* environmental fluctuations occur over a time span equal to or slightly longer than the lifespan of the organism, or when the organism is relatively sedentary in a heterogeneous environment and different selection pressures operate in different environmental patches. Short-term environmental fluctuations relative to the lifespan of the organism, or high mobility of an animal within a heterogeneous environment, or lack of discrimination between patches, favor phenotypic plasticity. Narrow habitat preferences correlate with specialized behavioral and morphological characteristics, and with the lack of either color polymorphism *or* phenotypic plasticity.

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SUMMARY

A case of shell color ploymorphism in the shallow water mesogastropod *Crepidula convexa* is described. Polymorphism of color in *C. convexa* appears to be based on disruptive selection, which occurs due to the availability of two contrasting shades of substrate, heterogeneous in time and space, combined with mortality from visual predators.

Data on the relative survival of the two phentoypes of *C. convexa* in two patches of a heterogeneous environment are applied to the fitness set model developed by Levins (1968). The necessary and sufficient criteria for a stable polymorphism are met, but reasons are set forth for believing that this is, rather, a case of unstable polymorphism.

Only one other species of the genus, *C. adunca*, shows a similar color polymorphism. Ecological factors uniquely shared by the two species are: visibility of inherited shell color, a major part of the population living in the intertidal zone, and an impact by visual predators. Color polymorphism is correlated with visual predation on a heterogenous substrate, and with low adult mobility. In general, the strategy of polymorphism is advantageous to some species of *Crepidula* and not others, depending on heterogeneity of the environment in time and space, with respect to its use by the organisms.

Other patterns of phenotypic variation found in *Crepidula* are phenotypic plasticity and phenotypic uniformity. Uniformity occurs when a species' behavior pattern restricts it to homogeneous environments. Plasticity, like polymorphism, increases niche size in heterogeneous environments; plasticity is favored when each organism must cope with environmental change within its lifetime. These patterns of natural selection for phenotypic variation probably apply to a wide variety of organisms.

LITERATURE CITED

BORKOWSKI, T. V., AND M. R. BORKOWSKI, 1969. *The Littorina ziczac* species complex. *Veliger,* **11**: 408–414.

CAIN, A. J., AND P. M. SHEPPARD, 1950. Selection in the polymorphic land snail *Cepaea nemoralis. Heredity,* **4**: 275–294.

Cain, A. J., and P. M. Sheppard, 1954. Natural selection in *Cepaea*. *Genetics*, **39**: 89–116.

Carriker, M., 1955. Critical review of the biology and control of oyster drills *Urosalpinx* and *Eupleura*. *U.S. Fish Wildl. Serv. Spec. Sci. Rep. Fish.*, **148**: 1–150.

Childress, J. R., 1972. Behavioral ecology and fitness theory in a tropical hermit crab. *Ecology*, **53**: 960–964.

Franz, D. R., and G. Hendler, 1970. Substrate diversity and the taxonomy of *Crepidula convexa* (Say) (Gastropoda: Prosobranchia). *Occas. Pap. Univ. Connecticut* (*Biol.*), **1**: 281–289.

Fretter, V., and A. Graham, 1962. *British prosobranch molluscs.* Ray Society, London, 755 pp.

Giesel, J. T., 1970. On the maintenance of a shell pattern and behavioral polymorphism in *Acmaea digitalis*, a limpet. *Evolution*, **24**: 98–119.

Gillespie, J., 1974. Polymorphism in patchy environments. *Am. Nat.*, **108**: 145–151.

Hoagland, K. E., 1974. Biological and physical causes of mortality in New England *Crepidula* species. *Am. Malacol. Union Inc. Bull.*, **39**: 14–17.

Hoagland, K. E., 1975. Reproductive strategies and evolution in the genus *Crepidula*. *Ph.D. thesis, Harvard University*, Cambridge, Massachusetts, 350 pp.

Hoagland, K. E., 1977. Systematic review of fossil and recent *Crepidula* and a discussion of evolution of the Calyptraeidae. *Malacologia*, **16**: in press.

Komai, T., and S. Emura, 1955. A study of population genetics on the polymorphic land snail *Bradybaena similaris*. *Evolution*, **9**: 400–418.

Levene, H., 1953. Genetic equilibrium when more than one ecological niche is available. *Am. Nat.*, **87**: 311–313.

Levins, R., 1968. *Evolution in changing environments: some theoretical explanations.* Princeton University Press, Princeton, New Jersey, 120 pp.

Maynard Smith, J., 1970. Genetic polymorphism in a varied environment. *Am. Nat.*, **104**: 487–490.

McNaughton, S. J., 1970. Fitness sets for *Typha*. *Am. Nat.*, **104**: 337–341.

Moore, H. B., 1936. The biology of *Purpura lapillus*. 1. Shell variation in relation to environment. *J. Mar. Biol. Assoc. U. K.*, **21**: 61–89.

Owen, G., 1966. Feeding. Pages 1–51 in K. M. Wilbur and C. M. Yonge, Ed., *Physiology of Mollusca*. Academic Press, New York and London.

Paine, R. T., 1969. The *Pisaster-Tegula* interaction: prey patches, predator food preferences, and intertidal community structure. *Ecology*, **50**: 950–961.

Verrill, A. E., and S. I. Smith, 1874. *Report upon the invertebrate animals of Vineyard Sound and adjacent waters, with an account of the physical features of the region.* Government Printing Office, Washington, D. C., 478 pp., 36 pls.

Reference: *Biol. Bull.*, **152**: 373–381. (June, 1977)

PARTIAL CHARACTERIZATION OF THE HEMOLYMPH LIPIDS OF *MERCENARIA MERCENARIA* (MOLLUSCA: BIVALVIA) BY THIN-LAYER CHROMATOGRAPHY AND ANALYSES OF SERUM FATTY ACIDS DURING STARVATION[1]

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Numerous studies have been made on the lipid composition of bivalve molluscs, particularly the sterols, although little information is available on bivalve lipid metabolism (Giese, 1966; Voogt, 1972). It is known that fat digestion in bivalves occurs extracellularly by means of lipases from the crystalline style (George, 1952; Patton and Quinn, 1973). Recently Cheng and Rodrick (1975) demonstrated low lipase activity in the hemolymph of *Crassostrea virginica* and *Mercenaria mercenaria;* and Cheng and Yoshino (1976) examined changes in lipase activity in the hemolymph cells, serum, and mantle fluid of *Mya arenaria* during phagocytosis. Since lipids are digested in the bivalve gut and lipases occur in the hemolymph, knowledge of the lipid content of the hemolymph would greatly aid an understanding of bivalve physiology, specifically the role of the hemolymph in lipid transport.

Lipids are interrelated by their fatty acids; therefore, changes in fatty acid profiles have been used to assess changes in lipid metabolism in a variety of invertebrates (Barrett, 1969; Hoskin, Cheng, and Shapiro, 1974; Pocock, Marsden, and Hamilton, 1971; Schaefer and Washino, 1969). The following investigation was undertaken to determine the neutral lipid composition of the serum and hemolymph cells of *Mercenaria mercenaria* and to examine the variation of serum fatty acid profiles among individual specimens. The reliability of using serum fatty acid profiles to indicate physiological condition resulting from starvation was also investigated.

MATERIALS AND METHODS

Animals

Specimens of the quahog clam, *Mercenaria mercenaria*, from upper Barnegat Bay, New Jersey, were used for analyses of hemolymph neutral lipids. The clams were purchased from a local supplier during the month of July. They were received and bled within 24 hours of digging. Anterior-posterior shell lengths of specimens used were between 7.8 and 8.3 cm. Analyses of serum fatty acids were made on specimens from Buzzards Bay, Massachusetts. They were received within 48 hours after digging. Shell lengths ranged between 8.8 and 10.0 cm. Sex of specimens was not determined. Initial fatty acid analyses were

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made on clams dug in March. Clams used in the starvation study were dug in June.

Hemolymph sampling

Hemolymph was obtained from the sinus of the posterior striated ("quick") adductor muscle using a technique modified after Feng, Feng, Burke, and Khairallah (1971). The shell was notched adjacent to the muscle, a syringe was inserted through the smooth ("catch") muscle into the striated muscle and a 3–4 ml hemolymph sample was slowly withdrawn. The hemolymph samples were immediately centrifuged at 9800 *g*-min to separate the cells from the serum.

For serum fatty acid analyses 1.5 ml hemolymph samples were taken from the specimens on the date of purchase. In the starvation studies specimens were then maintained at 12° C in aerated artificial sea water (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio). The salinity was 29‰. Hemolymph samples were taken after 5 days and 30 days.

To determine whether phytoplankton had grown in the aquarium from cells accidentally introduced with the clams, two 15 ml water samples were collected on the fifth and thirtieth days, centrifuged, and the sediment was examined by light microscopy. No phytoplankton were present, and almost no particulate matter of any kind was observed.

Thin-layer chromatography (TLC)

The lipids were extracted from each hemolymph cell and serum sample according to the procedure of Allen (1972). The extracting solvent consisted of the upper phase of a benzene/isopropanol/water (2/2/1) mixture. Lipases were inactivated by the isopropanol (Gurr and James, 1975).

Analytical grade isopropanol (Fisher Scientific Co., Fair Lawn, New Jersey) and Instra-Analyzed benzene (J. T. Baker Chemical Co., Phillipsburg, New Jersey) were used. All extractions were performed with 2 ml volumes and repeated once for a total of 4 ml. External standards consisting of 3 ml volumes of 0.9% NaCl plus 5 μl Hormel NL1 neutral lipid standard (Hormel Institute, Austin, Minnesota) were employed. These external standards contained 1 μg each of cholesterol, oleic acid, triolein, methyl oleate and cholesterol oleate. Reagent blank determinations were performed by extracting 3 ml samples of 0.9% NaCl. Glassware was cleaned as previously described (Hoskin *et al.*, 1974).

TLC analyses were performed on 20 × 20 cm silica gel sheets (Bakerflex IB2, J. T. Baker Chemical Co.). The sheets were predeveloped in chloroform/methanol (2/1) and dried. The sheets were dipped in freshly prepared 8% molybdosilicic acid (Climax Molybdenum Co., New York, New York) in 70% ethanol to about 0.5 cm above the sample application line. All dipped sheets were used within 4 hr.

The samples were applied to the TLC sheets with an automatic spotter (Chromaflex Spotter, Kontes Glass Co., Vineland, New Jersey) connected to a cylinder of dry nitrogen gas. The sample vials were rinsed with 0.2 ml solvent which was similarly applied. Resultant spot diameters ranged between 0.3–0.6 cm. The spots were concentrated into a line at the origin by development in benzene/isopropanol (2/1).

The sheets were developed 12 cm in filter paper lined TLC tanks equilibrated 30–40 min with petroleum ether/diethyl ether/acetic acid (85/15/1). The sheets were dried quickly with a hair dryer, then placed in a 100° C chromatography oven for 5 min. They were scanned within 2 hr with a densitometer (Chromaflex K495000, Kontes Glass Co.) connected to a 10 mV, 10 inch recorder (Model 1005, Beckman-Instruments Inc., Fullerton, California). Peak areas were determined by the method of height times width at half height (Johnson and Stocks, 1971). Peak areas of cell and serum lipid components were compared with peak areas from known amounts of Hormel NL1 standard. One lane of every TLC sheet was reserved for a 5 μl standard and a second lane for a reagent blank or external standard.

Serum cholesterol and triglyceride

Serum cholesterol and triglyceride levels were determined for three samples each consisting of serum pooled from three different specimens. Measurement of cholesterol oxidase activity provided values for total free and esterified cholesterol (Allain, Poon, Chan, Richmond, and Fu, 1974). A commercially available kit was adapted for these analyses (Cholesterol CHOD-PAP, Boehringer-Mannheim, Indianapolis, Indiana).

Triglyceride determinations were based upon total glycerol using a commercially available kit (Eskalab Reagent for Triglycerides, Smith Kline Instruments, Inc., Sunnyvale, California). Additional determinations were performed with an Automatic Clinical Analyser (DuPont Co. Instrument Products, Wilmington, Delaware).

Gas-liquid chromatography (GLC)

A chromatograph with on-column injection and dual flame ionization detectors was used (Model 3920, Perkin-Elmer Corp., Norwalk, Connecticut). Helium carrier gas flow rate was adjusted to 20 ml/min.

To determine the relative amount of cholesterol in the sterol fractions, blood was collected from two specimens, and the lipids from the sera and cells subjected to TLC as described above. Silica gel G on glass plates (Analtech Inc., Newark, New Jersey) was used because of the ease of removing the adsorbant following development. The gel containing sterol was scraped into glass vials, and the sterols were eluted with three washings of benzene, concentrated, and analyzed. Columns were 6 ft. stainless steel, 1/8 inch O.D., packed with 3% SP2250 on 100/120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pennsylvania). Cholesterol (Nutritional Biochemicals Co., Cleveland, Ohio) and a cholestane-cholesterol blend (Supelco, Inc.) were used as standards. The cholesterol peak was identified by comparison of absolute retention times of sample peaks with the standards.

Fatty acid methyl esters were prepared directly from 1 ml serum samples by a modification of the procedure of Ferguson (1975). Saponification was accomplished by adding 1 ml of 0.1 N KOH in methanol to each serum sample. The sample tubes were sealed and heated to 100° C in a water bath for 10 min. After the samples cooled, 1 ml of 14% methanolic boron trifluride was added, the tubes were heated at 100° C for 10 min, cooled, and the fatty acid methyl esters were extracted with three 1 ml volumes of hexane. One ml amounts of sterile 0.9% saline were identically treated to serve as controls.

The samples were concentrated to dryness under a stream of nitrogen gas, redissolved in 20 μl hexane, and analyzed. Columns were 6 ft. stainless steel, 1/8 inch O.D., packed with 10% SP216PS on 100/120 Supelcoport (Supelco, Inc.). Column temperature was 180° C. Tentative identification of peaks was made by comparison of absolute retention times with methyl esters of: $C_{10:0}$, $C_{12:0}$, $C_{13:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{17:0}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{19:0}$, $C_{20:0}$, $C_{20:1}$, $C_{20:2}$, $C_{20:3}$, $C_{20:5}$, $C_{21:0}$, $C_{22:0}$, $C_{22:1}$, $C_{22:5}$, $C_{22:6}$, $C_{24:1}$ (Supelco, Inc. or Applied Science Laboratories, Inc., State College, Pennsylvania). Peaks which did not correspond identically with any of the available standards were identified by use of a nomogram constructed from retention time data of the 23 standards according to the method of Haken and Souter (1966).

RESULTS

TLC analysis of neutral lipids

Retardation factors, (R_f) for the standards were: cholesterol, 0.09; oleic acid, 0.24; trioleate, 0.52; methyl oleate, 0.70; and cholesterol oleate, 0.88.

Results from the quantitative determination of twelve 5 μg standards applied to 10 TLC sheets indicate that the overall coefficient of variation (CV) was beween 7 and 12% (Table I). The efficiency of extraction of the 5 μg external standards from saline was within this experimental variation, so individual corrections for peak areas were not made. Sterols, nonesterified fatty acids, and sterol esters were present in both serum and cells (Table II). Triglycerides were prominent in cell extracts but were present only in trace amounts in serum samples. Fatty acid methyl esters were never detected in cells or serum. An unidentified lipid component with a R_f value of 0.82 was resolved in the blood cell lipid samples. This component was excluded from the value for total cell lipid.

Enzymatic determination of serum cholesterol and triglyceride

Total cholesterol levels for three pooled samples were 5.2 ± 0.4 mg/100 ml, 6.9 ± 0.7 mg/100 ml, and 7.5 ± 0.3 mg/100 ml.

Serum triglycerides were at the 1.0 mg/100 ml level of detection for the serum triglyceride analysis kit and below the level of detection of the automated pro-

TABLE I

Results of twelve 5 μl amounts of standard applied to ten TLC sheets, separated, then measured by densitometry. Each neutral lipid component is 1 μg.

Component	Peak areas (mm²) from densitometer tracings	Coefficient of variation
Cholesterol	160.0 ± 19.9	12.4%
Oleic acid	350.0 ± 23.7	6.8%
Triolein	466.8 ± 58.5	12.5%
Methyl oleate	520.5 ± 39.7	7.6%
Cholesterol oleate	643.1 ± 64.9	10.1%

Table II

TLC analyses of the neutral lipid composition of hemolymph from twelve specimens of Mercenaria mercenaria.

Hemolymph fraction	Sterols	Free fatty acids	Triglycerides	Sterol esters	Per cent total hemolymph lipids (cells plus serum)
Total cells/ml	76.7 ± 17.2%	5.0 ± 3.1%	8.3 ± 3.6%	10.0 ± 5.0%	60.7%
Serum (1 ml)	80.7 ± 24.0%	6.4 ± 3.0%	2.1 ± 1.3%	10.7 ± 9.0%	39.3%

cedure. Duplicate determinations of the three pooled samples yielded values of about 1.0 mg/100 ml. The values for total serum lipids were calculated from the per cent composition of total cholesterol as established by TLC and GLC. The total serum lipids were 44–64 mg/100 ml. Triglyceride comprised 2.1% of the total serum lipid or 0.9–1.3 mg/100 ml. Total cell lipid was 64–95 mg/cells/100 ml, of which triglyceride accounted for 5.3–7.9 mg/cells/100 ml.

GLC analyses of sterols

Eighteen components were detected in each of the serum sterol samples. Cholesterol, the second most abundant sterol, comprised approximately 13% of the total serum sterol fractions. Two components, including cholesterol, accounted for 30 and 35% of the total serum sterols in the two samples.

Sixteen components were resolved in the sterol class from the blood cells. Cholesterol was the most abundant single component in one sample, the second most abundant component in the other sample and accounted for 18% of the total in both samples.

GLC analyses of fatty acids

Twenty-two fatty acid methyl ester peaks were present in chromatograms of the eight specimens examined for serum total fatty acids. Five fatty acids ($C_{16:0}$, $C_{18:1}$, $C_{20:1}$, $C_{20:5}$, and $C_{22:6}$) accounted for 65% of the total serum fatty acids, and eleven fatty acids accounted for over 90% of the total.

Following Deans' (1968) recommendation, the per cent composition was recalculated based only on the major components (Table III). Fourteen fatty acids each had a retention time identical with a standard, the eight other fatty acids were identified by use of the nomogram.

The per cent composition of serum fatty acids was initially more varied in the clams used in the starvation study as shown by the larger standard deviations (Table IV). Since $C_{22:6}$ was not included the relative amounts of the individual fatty acids are proportionately greater than those in Table III. After five days of starvation standard deviations had increased for seven fatty acids when the pooled data were compared. Thus, the amounts of these fatty acids became more varied among the individual specimens as the experiment progressed.

The trends observed were an increase in the percentage of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$. The percentage of $C_{20:1}$ and $C_{20:5}$ decreased (Table II). After five

TABLE III

Serum fatty acids from eight specimens of Mercenaria mercenaria.

Peak number	Probable identity		Per cent composition among the 11 major acids
1	$C_{12:1}$	+	—
2	$C_{14:0}$	*	—
3	$C_{14:2}$	+	—
4	$C_{16:0}$	*	16.8 ± 1.9
5	$C_{16:1}$	*	3.7 ± 0.9
6	$C_{17:0}$	*	—
7	$C_{17:1}$	+	4.1 ± 0.7
8	$C_{18:0}$	*	5.4 ± 1.2
9	$C_{18:1}$	*	10.6 ± 1.4
10	$C_{18:2}$	*	—
11	$C_{20:0}$	*	—
12	$C_{20:1}$	*	9.6 ± 1.5
13	$C_{20:2} + C_{21:0}$	*	3.5 ± 1.0
14	$C_{21:1}$	+	—
15	$C_{22:0}$	*	5.4 ± 0.8
16	$C_{20:4}$	+	4.8 ± 0.5
17	$C_{20:5}$	*	19.9 ± 1.5
18	$C_{23:1}$	+	—
19	$C_{24:0}$	+	—
20	$C_{24:1} + C_{22:2}$	+	—
21	$C_{22:5}$	*	—
22	$C_{22:6}$	*	16.0 ± 1.9

* = identity based on retention time identical to standard.
+ = identity predicted from nomogram.

days of starvation only the change in $C_{18:0}$ met the criterion for statistical significance at the $\alpha = 0.05$ level of the Student's *t*-test. However, all of these changes were significant at the $\alpha = 0.05$ level following 30 days of starvation.

DISCUSSION

Both TLC and enzymatic procedures for determination of serum triglyceride indicated that this class is virtually absent, although TLC revealed that triglycerides were prominent in hemolymph cells. Since triglyceride is practically absent from the serum, lipid is transported primarily as sterol esters, nonesterfied fatty acids or as triglyceride within hemolymph cells, rather than as serum triglyceride.

Bayne (1973) reported total lipid levels between 20 and 80 mg/100 ml in pericardial fluid of *Mytilus edulis*. Our values for total serum neutral lipid, based upon total cholesterol and relative per cent composition of the other classes, are between 46 and 65 mg/100 ml.

The sterol composition of molluscs is complex (Patterson, Khalil, and Idler, 1975). We have not attempted to resolve and identify all the hemolymph sterols but have considered only cholesterol, since cholesterol is the major sterol of many Gastropoda and Bivalvia (Idler and Wiseman, 1971). Our GLC analyses of the relative amount of cholesterol in the sterol classes of the serum and hemolymph cell fractions have revealed that cholesterol is one of the most abundant, but not the single most abundant component. However, the entire sterol class accounts for the major portion of the total serum lipid.

The four major serum fatty acids, in order of decreasing abundance, were $C_{20:5}$, $C_{16:0}$, $C_{22:6}$ and $C_{18:1}$. These were also the four most abundant fatty acids in the tissues of several other marine bivalves (Kochi, 1975) and in *Mesodesma mactroides,* the yellow clam (de Moreno, Moreno, and Brenner, 1976).

Jefferies (1972) found that $C_{16:0}$, $C_{20:5}$, and $C_{22:6}$ were the three fatty acids in greatest amount in whole *Mercenaria mercenaria.* However, $C_{18:1}$ was not one of the four major fatty acids and, in fact, was about equal in amount to $C_{18:0}$. He found the fourth most abundant tissue fatty acid was $C_{20:1}$, whereas this was the fifth major serum fatty acid in specimens examined in this study.

In *M. mactroides,* the amount of $C_{18:1}$ showed a seasonal decrease which de Moreno *et al.* (1976) attributed to differences in the available phytoplankton food. Jefferies (1972) also found seasonal differences in two tissue fatty acids of *M. mercenaria,* $C_{20:5}$ and $C_{20:4}$.

The serum fatty acid profiles of specimens of *M. mercenaria* were remarkably consistent in the clams received in March but less consistent in those received in June. Whether the greater standard deviations of amounts of serum fatty acids in clams dug in June resulted from seasonal or other factors was not apparent.

Farrington, Quinn, and Davis (1973) reported differences in fatty acid composition of specific lipid classes of the polychaete, *Nephtys incisa,* and the bivalve *Yolida limatula.* They related these differences to pollution levels at the collecting sites. Jefferies (1972) included deviations in fatty acid profiles as part of a pollution induced "stress syndrome" in *M. mercenaria.* Although the basis for the effect of pollution on the fatty acid compositions of these organisms is not known, both pollution stress and starvation resulted in decreased $C_{20:5}$ in *M. mercenaria.*

Analyses of serum fatty acid profiles are attractive because they do not require destruction of the specimen tested. However, in this study they did not provide reliable evidence for the physiological condition of *M. mercenaria* during the first

Table IV

Per cent composition of ten serum fatty acids from five specimens of M. mercenaria *before and after starvation in artificial sea water.*

Peak number and tentative identity		Day 0	Day 5	Day 30
4	$C_{16:0}$	19.4 ± 3.5	22.6 ± 4.9	28.7 ± 6.7*
5	$C_{16:1}$	4.1 ± 1.0	4.9 ± 1.7	6.0 ± 1.7*
7	$C_{17:1}$	4.7 ± 1.2	3.7 ± 0.9	2.9 ± 1.7
8	$C_{18:0}$	6.0 ± 1.2	9.9 ± 1.1*	18.1 ± 3.9*
9	$C_{18:1}$	13.4 ± 1.8	18.2 ± 4.0	23.8 ± 6.1*
12	$C_{20:1}$	11.2 ± 2.2	10.9 ± 1.6	7.5 ± 1.2*
13	$C_{20:2} + C_{21:0}$	4.4 ± 0.7	2.0 ± 1.1	6.2 ± 1.6
15	$C_{22:0}$	6.1 ± 0.8	6.5 ± 1.6	4.4 ± 1.2
16	$C_{20:4}$	5.9 ± 0.6	6.0 ± 5.6	7.6 ± 5.5
17	$C_{20:5}$	24.9 ± 2.1	15.2 ± 6.8	4.7 ± 2.1*

* Mean different at $\alpha = 0.05$ level from the mean on day 0.

five days of starvation. After 30 days of starvation, relative amounts of six fatty acids were statistically different from the initial levels.

SUMMARY

TLC analysis of the neutral lipids in the hemolymph cells and serum of twelve specimens of *Mercenaria mercenaria* was performed. The serum fractions contained sterols (81%), free fatty acids (6%), sterol esters (11%), and a small amount of triglyceride (2%). Absolute amounts of chloesterol and triglyceride were 5.2–7.5 mg/100 ml and about 1 mg/100 ml, respectively. More total lipid and nearly all triglyceride was found to be in the cell fraction of the hemolymph. Cholesterol comprised 18% of the hemolymph cell sterols and 13% of the serum sterols.

The five major fatty acids, in order of decreasing abundance, in the sera of specimens of *Mercenaria mercenaria* harvested in March and June from Buzzards Bay, Massachusetts, were $C_{20:5}$, $C_{16:0}$, $C_{22:6}$, $C_{18:1}$, and $C_{20:1}$. A total of 22 fatty acids were resolved with tentative identification made for all of them. Standard deviations of the per cent composition of the major fatty acids were greater among specimens harvested in June than among those harvested in March.

The effect of starvation on the serum fatty acid profile of clams collected in June was determined. Starved specimens had relatively greater amounts of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ and reduced levels of $C_{20:1}$ and $C_{20:5}$. The $C_{20:5}$ was 20% lower on day 30 than day 0.

LITERATURE CITED

ALLAIN, C. C., L. S. POON, C. S. G. CHAN, W. RICHMOND, AND P. C. FU, 1974. Enzymatic determination of total serum cholesterol. *Clin. Chem.*, **20**: 470–475.

ALLEN, P. C., 1972. New extraction method for nematode lipids. *Anal. Biochem.*, **45**: 253–259.

BARRETT, J., 1969. The effect of ageing on the metabolism of the infective larvae of *Strongyloides ratti* Sandground, 1925. *Parasitology*, **59**: 3–17.

BAYNE, B. L., 1973. Physiological changes induced in *Mytilus edulis* L. induced by temperature and nutritive stress. *J. Mar. Biol. Assoc. U.K.*, **53**: 39–58.

CHENG, T. C., AND G. E. RODRICK, 1975. Lysomal and other enzymes in the hemolyph of *Crassostrea virginica* and *Mercenaria mercenaria*. *Comp. Biochem. Physiol.* **52B**: 443–447.

CHENG, T. C., AND T. P. YOSHINO, 1976. Lipase activity in the serum and hemolymph cells of the soft-shelled clam, *Mya arenaria,* during phagocytosis. *J. Invert. Pathol.*, **27**: 243–245.

DEANS, D. R., 1968. Accurate quantitative gas-chromatographic analysis—I. Methods of calculating results. *Chromatographia*, **1**: 187–194.

FARRINGTON, J. W., J. G. QUINN AND W. R. DAVIS, 1973. Fatty acid composition of *Nephtys incisa* and *Yolida limatula*. *J. Fish. Res. Board Can.*, **30**: 181–185.

FENG, S. Y., J. S. FENG, C. N. BURKE AND L. H. KHAIRALLAH, 1971. Light and electron microscopy of the leucocytes of *Crassostrea virginica* (Mollusca: Pelecypoda). *Z. Zellforsch. Mikrosk. Anat.*, **120**: 222–245.

FERGUSON, J. C., 1975. Fatty acid and carbohydrate storage in the annual reproductive cycle of *Echinaster*. *Comp. Biochem. Physiol.*, **52A**: 585–590.

GEORGE, W. C., 1952. The digestion and absorption of fat in lamellibranchs. *Biol. Bull.*, **102**: 118–127.

GIESE, A. C., 1966. Lipids in the economy of marine invertebrates. *Physiol. Rev.*, **46**: 244–298.

GURR, M. I., AND A. T. JAMES, 1975. *Lipid biochemistry: an introduction,* 2nd ed. Chapman and Hall, London, 244 pp.

Haken, J. K., and P. Souter, 1966. Evaluation of retention data of compounds classifiable into homologous series. *J. Gas Chrom.*, **4**: 295–299.

Hoskin, G. P., T. C. Cheng and I. L. Shapiro, 1974. Fatty acid compositions of three lipid classes of *Himasthla quissetensis* rediae before and after starvation. *Comp. Biochem. Physiol.*, **47B**: 821–830.

Idler, D. R., and P. Wiseman, 1971. Sterols of molluscs. *Int. J. Biochem.* **2(11)**: 516–528.

Jefferies, H. P., 1972. A stress syndrome in the hard clam, *Mercenaria mercenaria*. *J. Invert. Pathol.*, **20**: 242–251.

Johnson, A. R., and R. B. Stocks, 1971. Gas-liquid chromatography of lipids. Pages 199–218 in A. R. Johnson and J. B. Davenport, Eds., *Biochemistry and methodology of lipids.* Wiley, New York.

Kochi, M., 1975. Fatty acid composition of lipids from some marine shellfish. *Suisan Daigakko Kenkyu Hokoku*, **23(5)**: 155–162.

de Moreno, J. E. A., V. J. Moreno, and R. R. Brenner, 1976. Lipid metabolism of the yellow clam, *Mesodesma mactroides:* I. Composition of the lipids. *Lipids*, **2**: 334–340.

Patterson, G. W., M. W. Khalil, and D. R. Idler, 1975. Sterols of Scallop: I. Application of hydrophobic Sephadex derivatives to the resolution of a complex mixture of marine sterols. *J. Chromatogr.*, **115**: 153–159.

Patton, J. S., and J. G. Quinn, 1973. Studies on the digestive lipase of the surf clam *Spisula solidissima*. *Mar. Biol.*, **21**: 59–69.

Pocock, D. M.-E., J. R. Marsden, and J. G. Hamilton, 1971. Lipids in an intertidal polychaet and their relation to maturation of the worm. *Comp. Biochem. Physiol.*, **39A**: 683–697.

Schaefer, C. H., and R. K. Washino, 1969. Changes in the composition of lipids and fatty acids in adult *Culex tarsalis* and *Anopheles freeborni* during the over-wintering period. *J. Insect Physiol.*, **15**: 395–402.

Voogt, P. A., 1972. Lipid and sterol components and metabolism in Mollusca. Pages 245–300. in M. Florkin and B. T. Scheer, Eds., *Chemical zoology, Vol. VII, Mollusca.* Academic Press, New York.

Reference: *Biol. Bull.*, **152**: 382–391. (June, 1977)

DEVELOPMENT OF THE DIMORPHIC CLAW CLOSER MUSCLES OF THE LOBSTER, *HOMARUS AMERICANUS*:
II. DISTRIBUTION OF MUSCLE FIBER TYPES IN LARVAL FORMS

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The bilateral closer muscles in the dimorphic claws of adult lobsters are unusual in that, like the claws, they are asymmetrical (Jahromi and Atwood, 1971; Goudey and Lang, 1974; Lang, Costello and Govind, 1977). The crusher claw closer muscle is composed solely of long sarcomere slow fibers. In the cutter claw, the closer muscle contains both fast and slow muscle fibers. This results in a bimodal distribution of muscle fiber types in the cutter (based on sarcomere length), with over 60% being short sarcomere, fast fibers and the remainder being long sarcomere, slow fibers. In the cutter closer muscle, fast and slow fibers are regionally distributed on the inner aspect with fast fibers in the dorsal and central medial sections and slow fibers in the ventral section (Lang *et al.*, 1977).

An interesting feature of the claws is that while they are asymmetric in the adult, they are symmetric during the larval and early postlarval period. Thus in the larval forms, which comprise the first three stages, the bilateral chelipeds are identical, both being cutter-like in appearance. The transformation of one claw of the pair to a crusher cheliped occurs later in development, during the seventh or eighth stage (Herrick, 1896). Presumably, the differentiation of the closer muscle into cutter and crusher types is associated with the change in external morphology of the cheliped which occurs during this time.

The present paper reports on the composition of the bilateral closer muscles in the three larval stages. In each larval stage the bilateral muscles are similar in fiber composition, each having in the first stage mainly short and intermediate sarcomere fibers and relatively few long sarcomere fibers. In the second and third larval stages the closer muscles have an equal distribution of all three fiber types.

Materials and Methods

Newly hatched lobsters were obtained from the Massachusetts State Hatchery on Martha's Vineyard and reared in circulating seawater tanks at ambient temperature (Hughes, Shleser and Tchobanoglous, 1974). Animals were fed brine shrimp several times a day. The first three stages are larval, and during this time the animals are pelagic. Each of the larval stages is easily identifiable by size and external morphology (Herrick, 1896, 1911).

The closer muscle in the claw was prepared as follows: the limb was pinned in a wax bottom dish with the dactyl in the fully open position so that the closer muscle fibers were held in the stretched position. To permit a constant flow of fixative past the closer muscle, the limb was cut in the carpodite and in the propodite distal to the muscle. The limb was immersed in freshly prepared aqueous Bouin's and a fine jet of the fixative was directed at the cut ends during the first 10 min. After 24 hr in the fixative, the closer muscle was isolated from the limb and stored in 70% alcohol.

The inner aspect of each closer muscle was divided into sections to determine whether there was any regional distribution of fiber types, as there is in the adult (Lang *et al.*, 1977). In the second and third stages, the claw was divided into quadrants and muscle fibers were sampled from each of the four sections. The first stage claw was too small to be similarly divided so fibers were sampled from two areas, dorsal and ventral, for this stage. The method for obtaining the average sarcomere length of a muscle fiber is described in an earlier paper (Lang *et al.*, 1977).

Muscle fiber populations were compared for significant differences (at the 0.05 and 0.01 levels) using the Kolmogorov-Smirnoff two-sample test (Siegel, 1956). Comparisions were made between the two claws for each animal and between the total claw I and claw II populations for each of the four stages examined (stages 1, 2, 3, and late 3). Differences between stages were tested using the total claw I and total claw II populations for each stage.

RESULTS

The paired claw closer muscles in each of the three larval stages of the lobster, *Homarus americanus*, were examined to determine the muscle fiber types that compose them. Using the average sarcomere length of a fiber as a criterion, three fiber types were recognized: short, intermediate and long sarcomere fibers with sarcomere lengths of < 4, 4–6, and > 6 μm, respectively. Table 1 gives the relative distribution of fiber types in the bilateral closer muscles, and lists the

TABLE I

Distribution of muscle fiber types in the paired claw closer muscles of larval lobsters.

Stage	Length (cm)	Muscle fiber types based on sarcomere length (μm)					
		Claw I			Claw II		
		Short <4	Intermediate 4–6	Long >6	Short <4	Intermediate 4–6	Long >6
1	0.7	47%	53%	0%	27%	73%	0%
1	0.75	37	57	6	30	60	10
1	0.75	33	63	4	30	70	0
2	0.8	47.5	50	2.5	42.5	52.5	5
2	0.85	42.5	57.5	0	40	60	0
2	0.85	40	57.5	2.5	37.5	55	7.5
3	1	20	60	20	5	75	20
3	1	32.5	55	12.5	30	52.5	17.5
3	1	35	52.5	12.5	17.5	62.5	20
3 (late)	1.05	40	20	40	35	47.5	17.5
3 (late)	1.2	40	37.5	22.5	35	32.5	32.5

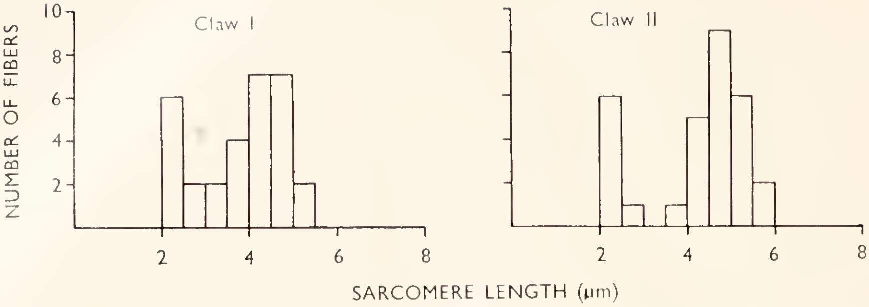

FIGURE 1. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of the paired closer muscles of a larval first stage lobster.

closer muscle with the higher percentage of short sarcomere fibers as belonging to claw I. Each larval stage will be described separately.

It should be noted that the muscle fibers in larval claws were classified, on the basis of sarcomere length, into categories similar to those used for the adult claw. However, we presently have no information regarding their physiological and biochemical characteristics. Thus for the larval stages we do not yet know whether the long sarcomere fibers contract more slowly than the short sarcomere fibers. For this reason fibers will be classified simply on the basis of sarcomere length.

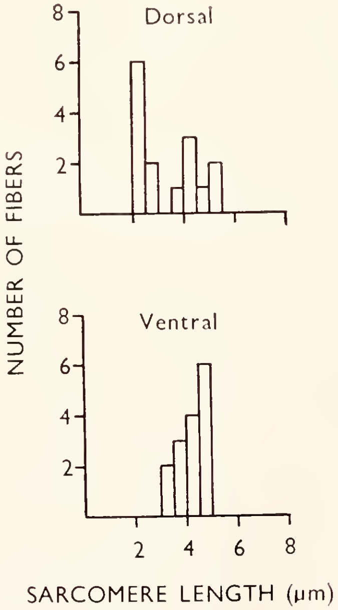

FIGURE 2. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution on the inner aspect of a Claw I closer muscle in a larval first stage lobster.

Stage 1

In first-stage lobsters the small size of the closer muscle (propus length *ca.* 0.9 mm) limited division of the muscle into two sections, dorsal and ventral. From each section 15 fibers were sampled for a total of 30 fibers for each muscle. The relative distribution of fiber types for the three pairs of stage 1 muscles is given in Table I. Each closer muscle of a pair is composed largely of intermediate and short sarcomere fibers and has either few or no long sarcomere fibers. This bimodal pattern is also reflected in a frequency histogram of sarcomere lengths in one muscle pair (Fig. 1) in which peaks typically occur at 2 μm and 4–5 μm. However, when the frequency of fiber types is plotted for the separate muscle areas from which they were sampled (Fig. 2), either the majority (Claw I) or all (Claw II) of the short sarcomere fibers are in the dorsal section. This is similar to the pattern found in the adult cutter closer muscle where the ventral section contains only long sarcomere slow fibers and the dorsal sections primarily short sarcomere fast fibers (Lang *et al.*, 1977).

Statistical comparisons of the claw muscle fiber populations did not reveal any differences between Claw I and Claw II populations. This was true for comparison between claws of each animal as well as between summed Claw I and Claw II populations for three animals (Kolmogorov-Smirnoff two sample test, at 0.05 level).

Stage 2

In stage 2 larval lobsters the propus increases about 30% in length to 1.2 mm; thus, the inner side of the closer muscle could be divided into quadrants. Then fibers were sampled from each of the four sections. The closer muscle at this stage resembles its stage 1 counterpart in that each muscle is composed almost entirely of intermediate and short sarcomere fibers; long sarcomere fibers are entirely absent or, if present, are few in number (Table I). Likewise, each muscle shows a bimodal distribution of fiber types with peaks at 2 μm and

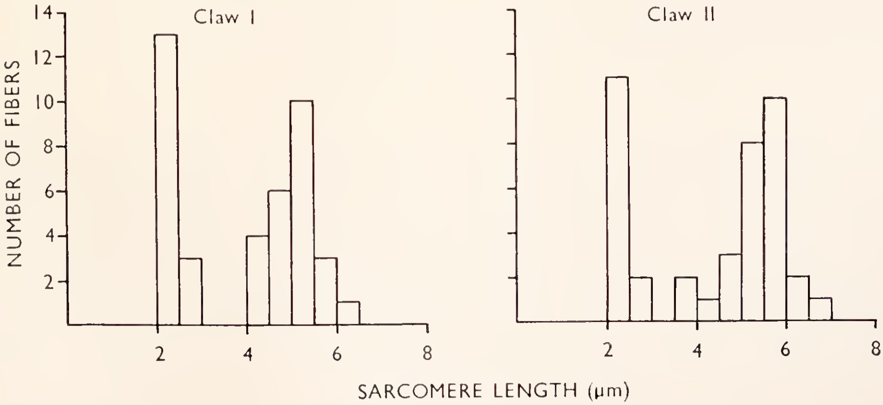

FIGURE 3. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of the paired closer muscles of a larval second stage lobster.

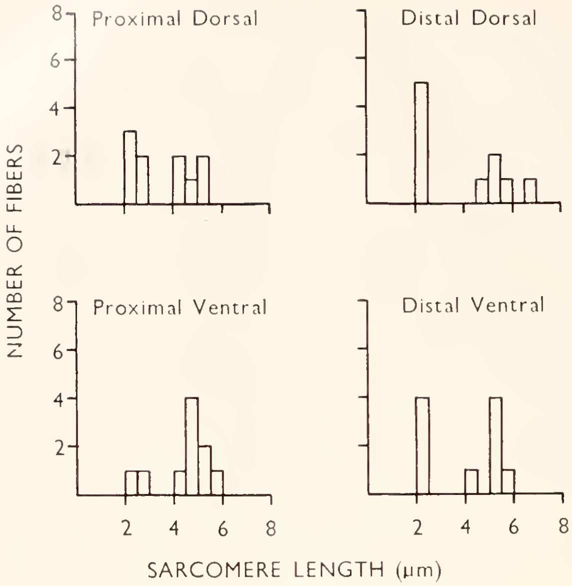

FIGURE 4. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution on the inner aspect of a Claw I closer muscle in a larval second stage lobster.

4.5 μm (Fig. 3). The fiber types do not appear to be regionally distributed, as both types occur in all four sections sampled (Fig. 4). Yet there may be a tendency for the proximal ventral section to contain only intermediate fibers (Fig. 4) as half the closer muscle samples were thus characterized.

Statistical comparisons of claw muscle fiber populations did not reveal any differences between Claw I and Claw II, either for a single animal or for the summed populations of the three animals. Likewise there was no significant difference (at the 0.05 level) between the Claw I and Claw II populations of stage 2 as compared to the respective populations of stage 1.

Stage 3

The sampling procedure for stage 3 closer muscles was similar to that followed for stage 2 muscles. The propus length measures approximately 1.9 mm in stage 3 animals. A total of five animals was examined in this larval stage: three animals were sacrificed when they were midway through this stage and the remaining two when they were near the end of this stage (late 3rd stage in Table I).

The 3rd stage closer muscle differs from its counterpart in the earlier stages in two respects. First, there is an increase in the number of long sarcomere fibers which now comprise between 12.5% and 40% in stage 3 as compared to < 10% in stages 1 and 2 (Table I). This increase is primarily at the expense of the short sarcomere fiber population. Secondly, although there is still a bimodal distribution of fiber types in stage 3 muscles, the second peak (intermediate fibers) now occurs at 5.5 μm (Fig. 5) as compared to 4.5 μm in the two earlier stages (Figs. 1, 3). The first peak (short sarcomere fibers) oocurs at 2 μm in stage 3 muscle (Fig. 5) as in the earlier stages.

A plot of the fiber types in the four sections reveals that all three fiber types occur consistently in the dorsal sections (Fig. 6). In the ventral sections, however, only the intermediate and long sarcomere fiber types occur regularly. There were one or fewer short sarcomere fibers (< 4 μm) present in the ventral sections in three out of the six claws examined. In addition, they comprised 9% of the ventral fiber population but 38% of the dorsal fiber population.

There were no significant differences between Claw I and Claw II populations for individual animals or for the summed population of the three samples. However, the summed population of both Claw I and Claw II were significantly different (at the 0.01 level) from the respective population of stage 2.

Stage 3 (late)

In the late third stage closer muscle, the relative proportions of the three fiber types change so that there is an increase in both short and long sarcomere fibers and a corresponding decrease in intermediate fibers (Table I). The change in the short sarcomere fibers is reflected by their increased occurrence in the ventral areas, while the long sarcomere fibers were observed in higher numbers in all areas. Whether this change in distribution, as compared to earlier third stage animals, is due to the premolt condition or whether it is due to a normal change in fiber types is uncertain. However, the summed Claw I and Claw II populations are significantly different (at the 0.01 level) from their respective stage 3 populations.

DISCUSSION

Neither the claws nor the claw closer muscles in the larval stages of the lobster are differentiated into cutter and crusher types. Both claw closer muscles have short, intermediate and long sarcomere muscle fibers. In the adult, the cutter

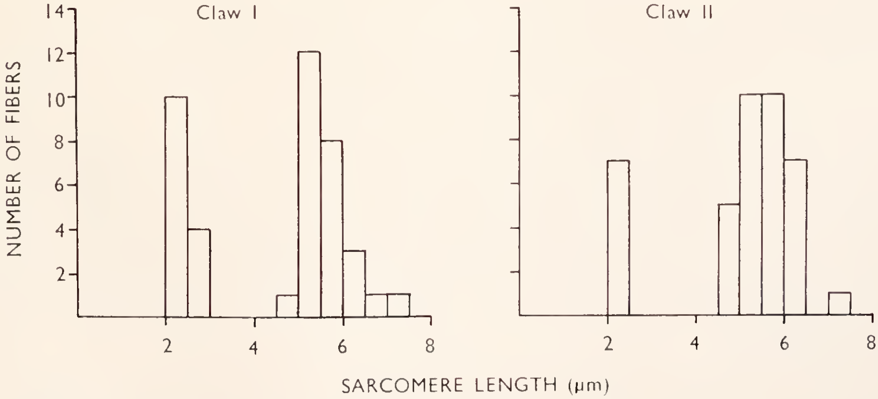

FIGURE 5. Frequency histogram of muscle fibers with characteristic sarcomere lengths from the inner aspect of the paired closer muscles of a larval third stage lobster.

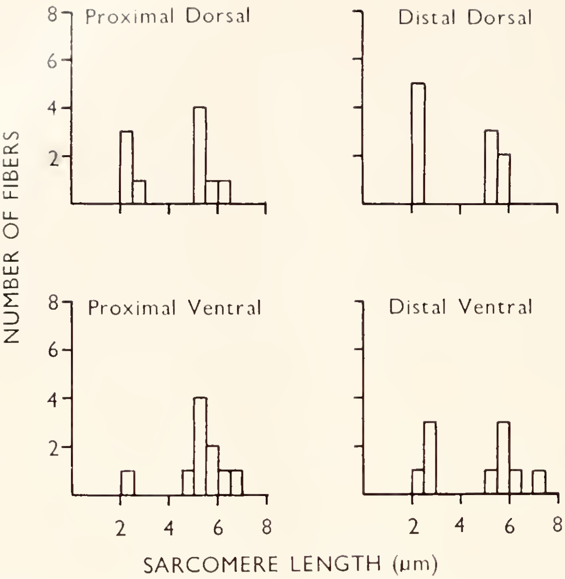

FIGURE 6. Frequency histogram of muscle fiber types (based on sarcomere lengths) showing the regional distribution on the inner aspect of a Claw I closer muscle in a larval third stage lobster.

claw has 60–70% short sarcomere fast fibers and 30–40% long sarcomere slow fibers, while the crusher has all long sarcomere slow fibers; a small proportion (2–4%) of intermediate sarcomere fibers are occasionally found in both muscles (Lang *et al.*, 1977). In contrast the larval muscles possess over 50% ($\bar{X} = 60\%$) intermediate fibers, except in the late third stage where there is a decrease to an average of 34% (Table I). There are few long sarcomere fibers ($\bar{X} = 3\%$) in the first two larval stages, but these increase up to an average of 28% in the late third stage. Short sarcomere fibers, on the other hand, average 38% of the population in the first two larval stages. During the middle of the third stage the short sarcomere fiber population is only 23% of the total, but during the late third stage it again reaches 38% of the total.

The major changes which occur during the larval stages are the transient decrease in the number of short sarcomere fibers in the mid-third stage and the accompanying increase in long sarcomere fibers. During late third stage the short sarcomere fiber population continues to become larger, both these changes being at the expense of the declining intermediate fiber population. From this larval condition, the transformation into the adult state involves the further acquisition of short and long sarcomere fibers only and the concomitant disappearance of the intermediate fibers.

How are these "new" short and long sarcomere fibers acquired? It is possible that they arise *de novo* as fully differentiated short and long sarcomere fibers, or alternatively that they differentiate from existing fibers, including especially the intermediate fibers. On the other hand, it is possible that they arise simply by differential proliferation of the existing populations of short and long sarcomere fibers, perhaps by fiber splitting. In regard to the first possibility, the abdominal extensor muscles which have morphologically distinct bundles of short sarcomere fibers and long sarcomere fibers exhibit the dichotomy in the earliest stage examined, *i.e.*, in larval lobsters and newly hatched juvenile crayfish (Govind, Atwood and Lang, 1974). However, the sarcomere lengths in these early stages are considerably smaller than those in the adult. Thus, though sarcomere length does increase during development, the differentiation into short sarcomere fibers and long sarcomere fibers is established very early. This apparently does not occur in the claw closer muscles; in the larval stages these muscles are symmetrical and composed of a mixed population of short, intermediate and long sarcomere fibers while the adult closer muscles are asymmetrical, there being a complete loss of short sarcomere fibers in the crusher claw. Thus, while fiber properties of the abdominal muscles are established and fixed during myogenesis, those of the claw muscle are not.

A corollary to this mechanism of genetically specified fiber types would necessitate the loss of the intermediate fibers typically found in the larval forms (Table I) rather than their transformation into short or long sarcomere type. Yet in all the larval lobsters examined in this study, no signs of degenerating muscle fibers were found. Also, the gradual increase in number of long fibers until the late third stage, associated with the shift in sarcomere length peak from 4.5 μm to 5.5 μm in the third larval stage, suggests that the intermediate fibers may transform to long sarcomere fibers by lengthening of their sarcomeres. In fact, the sarcomere length of the long fibers of the abdominal extensor muscles in larval lobsters measures 4–5 μm and subsequently lengthens to the adult size (Govind *et al.*, 1974). In this regard, it is of interest to note that while there is little change in the short sarcomere fiber population from the third to the fourth stage, there is a change in the other fiber populations. Fourth (first juvenile) stage claws average fewer than 5% intermediate fibers. Thus, there appears to be a further shift of the intermediate fibers to long sarcomere fibers at or shortly following the fourth molt (to the fourth stage) (in preparation). This problem of transformation of muscle fiber types does not appear to have been previously studied in invertebrate muscle, but it has been shown to occur in mammalian and avian muscle (for reviews see Harris, 1974; Gutmann, 1976).

The data support the hypothesis that there is a transformation of intermediate sarcomere fibers to long sarcomere fibers. Although care was taken to fix all claws in the same fully open position, it is possible that some differences were due to anatomical changes in the claw. This seems improbable for the changes observed between mid-third and late-third stage. Claw shape seems unlikely to change during the intermolt period, yet there was a statistically significant difference between claws from these two ages. However, definitive proof must await ultrastructural studies which will permit analysis of other characteristics of the muscle fibers including sarcomere length, A-band length, sarcoplasmic reticulum, etc.

While there may be transformation of intermediate fibers to long sarcomere fibers, it would appear that the same is not true in regard to transformation of intermediate fibers to short sarcomere fibers. If intermediate sarcomeres did transform by decrease of sarcomere lengh, it would also necessitate addition of sarcomeres to maintain a constant muscle fiber length. Furthermore, in the majority of larval muscles the short sarcomere fiber population is distinctly separated from the intermediate fiber population. The short sarcomere fibers range between 2–3.5 μm with the majority at 2 μm; the intermediate fibers range between 4–6 μm. If the intermediate fibers were redifferentiating into short sarcomere fibers, a continuous range between these fiber types (2–6 μm) without any distinct breaks might be expected.

Thus, the present data support the hypothesis for transformation of intermediate fibers to long sarcomere fibers. These two fiber types form a continuous population with no apparent bimodal distribution. However, it is clearly desirable to have ultrastructural evidence of these fiber populations, to unequivocally determine that they form a continuum.

We thank John Hughes, Director of the Massachusetts State Lobster Hatchery, Martha's Vineyard, for generously supplying us with larval lobsters and advising us in their maintenance. This work was supported by grants from: NIH and Muscular Dystrophy Association of America to F. L. and by NRC and Muscular Dystrophy Association of Canada to C. K. G.

SUMMARY

1. The closer muscles of the paired claws (chelipeds) of lobsters were characterized according to the distribution of short, long and intermediate sarcomere muscle fibers during the three larval stages.

2. Unlike the adult lobster, where the claws and closer muscles are asymmetrical, the claws and closer muscles of the larval stages are symmetrical.

3. In the first and second larval stages, the closer muscle is composed of over 50% intermediate sarcomere fibers, 30–40% short sarcomere fibers and less than 10% long sarcomere fibers.

4. By the late third stage the long sarcomere fibers have increased to a maximum of 40% with a corresponding decrease in number of intermediate fibers.

5. Thus, at the end of the last larval stage, the closer muscles are symmetrical, with muscle fibers about equally distributed among short, intermediate and long sarcomere fiber types.

6. The data are consistent with the hypothesis that intermediate fibers are transformed into long sarcomere fibers but not into short sarcomere fibers.

LITERATURE CITED

GOUDEY, L. R., AND F. LANG, 1974. Growth of crustacean muscle: asymmetric development of the claw closer muscles in the lobster, *Homarus americanus*. *J. Exp. Zool.*, **189**: 421–427.

GOVIND, C. K., H. L. ATWOOD, AND F. LANG, 1974. Sarcomere length increases in developing crustacean muscle. *J. Exp. Zool.*, **189**: 395–400.

GUTMANN, E., 1976. Neurotrophic relations. *Annu. Rev. Physiol.*, **38**: 177–216.

HARRIS, A. J., 1974. Inductive functions of the nervous system. *Annu. Rev. Physiol.*, **36**: 251–305.

Herrick, F. H., 1896. The American lobster: a study of its habits and development. *Bull. U. S. Fish Comm.*, **15**: 1–252.

Herrick, F. H., 1911. Natural history of the American lobster. *U. S. Bur. Fish.*, **29**: 149–408.

Hughes, J. T., R. A. Shleser, and G. Tchobanoglous, 1974. A rearing tank for lobster larvae and other aquatic species. *Prog. Fish.-Cult.*, **36**: 129–132.

Jahromi, S. S., and H. L. Atwood, 1971. Structural and contractile properties of lobster leg-muscle fibers. *J. Exp. Zool.*, **176**: 475–486.

Lang, F., W. J. Costello, and C. K. Govind, 1977. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus:* I. Regional distribution of muscle fiber types in adults. *Biol. Bull.*, **152**: 75–83.

Siegel, S., 1956. *Nonparametric statistics.* McGraw Hill Book Co., New York, 312 pp.

Reference: *Biol. Bull.*, **152**: 392–405. (June, 1977)

THE PTYCHOCYST, A MAJOR NEW CATEGORY OF CNIDA USED IN TUBE CONSTRUCTION BY A CERIANTHID ANEMONE

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The cerianthid anemones characteristically are burrowing forms, living in a tube of their own construction. It has been known for many years now that the tube of cerianthids is at least partially composed of discharged nematocysts along with various "foreign objects" including substrate material from the vicinity of the tube (*e.g.*, McMurrich, 1890; Hyman, 1940; Frey, 1970). However, no one to date appears to have examined critically the mechanism of tube formation in cerianthid cnidarians, nor the type(s) of nematocysts and the extent to which they might be involved. The general sentiment regarding this problem was probably best summarized by Torrey and Kleeberger (1909, p. 117) who stated, "There is little to be said of the sheath with which the animal readily surrounds itself. It serves as a lining for the burrow, is composed of a feltwork of nematocysts and is easily torn."

Carlgren (1912, p. 28) mentioned the extremely large nematocysts (up to 103 μm capsule length) with the "coiled spiral thread" which were extremely common in the column ectoderm of various cerianthids. Carlgren (1940, p. 15) later identifies these as "atrichs" and indicates they are the most numerous cnidae present on the column of cerianthids and that both holotrichs and spirocysts are rare in the same region. More recently, Schmidt (1972, 1974) has identified the nematocysts in the tubes of cerianthids as "atrichs."

In the present study, the so-called atrichous isorhiza (*i.e.*, atrich) involved in tube formation by a cerianthid anemone has been examined using a variety of microscopical methods. Rather than being an atrich, this cnida represents an entirely new, major category of coelenterate organelle which has heretofore been undescribed. Contrary to all nematocysts and spirocysts studied to date, this new cnida is not helically folded within the capsule prior to discharge. Instead, it exhibits an entirely new method of folding of the undischarged thread resulting in a variable number of pleats in circumference, but entirely lacking pleats in length.

MATERIALS AND METHODS

The species examined in the present study was *Ceriantheopsis americanus* Verrill, 1864) collected from the Alligator Point area of the north Florida Gulf Coast. For scanning electron microscopy (SEM), an entire *Ceriantheopsis* with a freshly formed tube was fixed in Parducz's (1967) fixative for one hour. Following fixation, a portion of the column containing the newly formed tube was excised and transferred to a 16% glycerol solution for 24 hours and then prepared for Freon critical point drying following the procedures given in Mariscal (1974a, b). The prepared tissues were then mounted, coated with gold-palladium in a Denton vacuum evaporator and examined in a Cambridge S4-10 scanning electron microscope.

For transmission electron microscopy (TEM), small portions of the mid-column epidermis in the region of tube formation were dissected out and this plus freshly secreted tube material were fixed in cold 2% glutaraldehyde in sea water for one hour, post-fixed in 2% OsO_4 in sea water for 30 minutes and stained in 2% uranyl acetate for 5 minutes. Following dehydration in a graded acetone series, the material was embedded in Epon, thin sectioned and examined in a Phillips 200 transmission electron microscope.

Light microscopy was done on a Reichert Zetopan phase contrast microscope with a Nikon AFM photomicrographic attachment.

Results

Tube construction

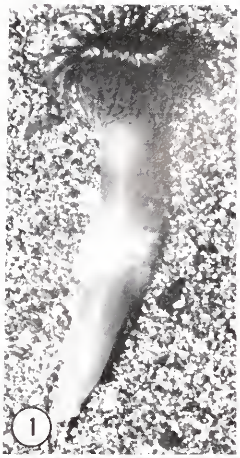
Within minutes of removing *C. americanus* from its tube, one can notice the formation of a fine, white sheath, representing the incipient new tube, enveloping the exposed column ectoderm (Fig. 1). About five minutes after tube removal, it is possible to see long, white filaments radiating out from the column ectoderm (Fig. 1). Examination of these filaments as well as the newly formed tube material under the light microscope showed a dense mat of the intertwined threads and capsules of thousands of discharged cnidae (Figs. 2, 3). Although several types of typical nematocysts (including holotrichous isorhizas and microbasic b-mastigophores) and spirocysts were present in the tube material, by far the vast majority of the cnidae present belonged to a new, undescribed category to which has been given the name of "ptychocyst."

Ptychocyst diagnosis

Undischarged thread not helically folded, with a variable number of pleats in circumference but no pleats in length; discharged thread long, non-isodiametric and with a variable number of fine ridges running along its length representing the folds by which it is compressed in diameter prior to discharge; thread tip closed and entire thread lacking in spines or hollow tubules; undischarged and partially discharged threads both basophilic and acidophilic. Name derived from Greek *ptychos* meaning fold.

Dimensions of capsule and thread

The ptychocysts are extremely variable in size, but are much larger than the spirocysts or mastigophores present. Carlgren (1912) mentions that the capsule of this cnida reaches a length of 70 μm and later (1940) gives the size range for apparently the same cnida (which he calls an "atrich") as being 36–82 μm in length and 12–25 μm in width. Measurements of ptychocysts in the present study revealed a range of 29–75 μm in length and 8–36 μm in width. However, there appeared to be two distinct size classes with the largest yielding measurements of 59–75 μm × 19–36 μm and the smallest being 29–36 μm × 8–12 μm in size.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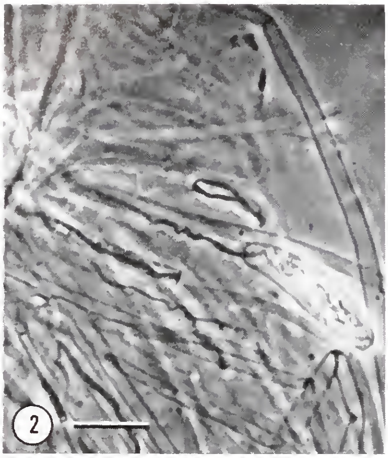
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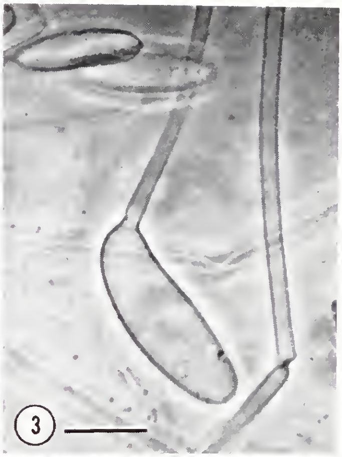
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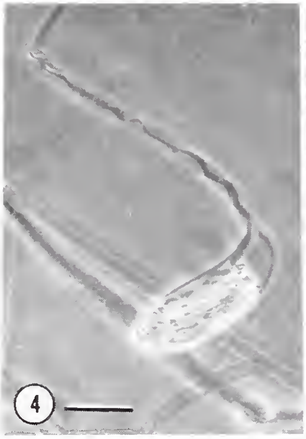
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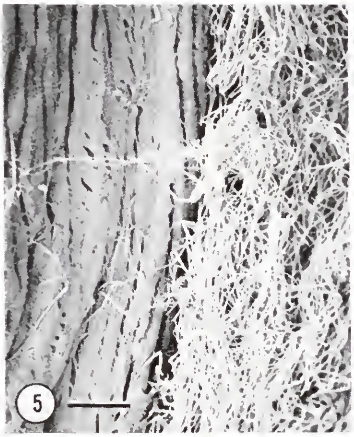
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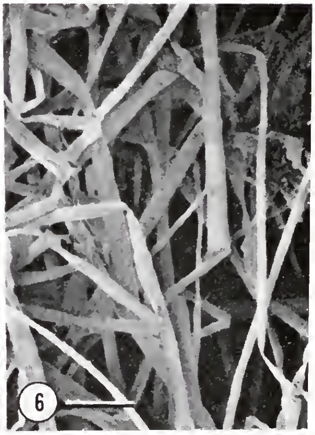
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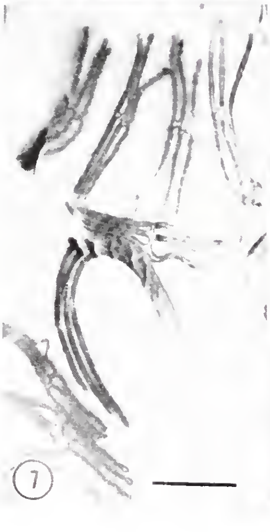
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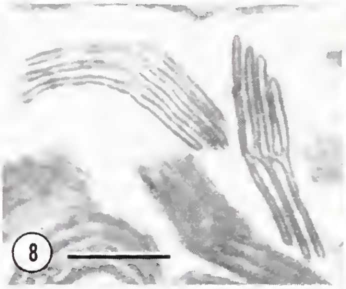
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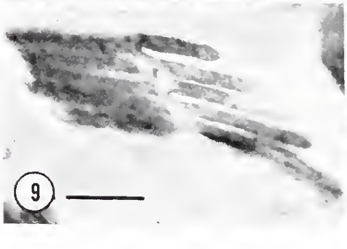
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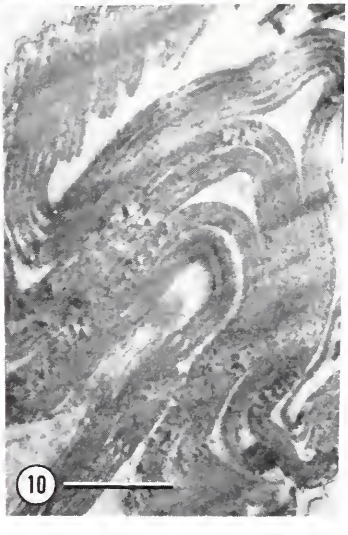
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Measurements of 20 cnidae in each size class gave mean values of 65 μm in length × 27 μm in width for the largest examples and 32 μm × 10 μm for the smallest ones. In other cerianthids, what appears to be this same cnida may be even larger since Carlgren (1940) mentions that the "atrichs" on the column of *Pachycerianthus multiplicatus* had a size of 113 × 43 μm, making them among the largest cnidae known.

The capsules of ptychocysts, with widths varying from 25% to 55% of the capsule length, tend to be broader than those of many anthozoan cnidae. However, the capsules appear to be bilaterally compressed, at least in the larger cnidae, and thus the above range of measurements probably reflects the orientation of the capsule under the microscope at the time of measurement.

The threads of ptychocysts are non-isodiametric and in large examples taper from about 5 μm near the capsule to about 2 μm at the closed tip for a total reduction in diameter of some 2.5 times. The discharged threads may be extremely long (over 2 mm in length) and with the naked eye can be seen projecting outwards from the column ectoderm during new tube formation (Fig. 1).

Mechanism of discharge and general characteristics of the thread

Like all nematocysts and spirocysts examined to date (*e.g.*, see Mariscal, 1974c and Mariscal and McLean, 1976), ptychocysts evert during discharge (Fig. 4). Although some threads appear to emerge more or less in line with the capsule, many others evert at an angle of about 60 to 90 degrees with the plane of the capsule (*e.g.*, Figs. 3, 4). If the capsules are oriented perpendicular to the column surface (as they appear to be), this is perhaps an adpatation to facilitate the intertwining of the discharging threads at various levels above the column surface in order to form a stronger, more tightly interwoven tube. The newly everted threads are apparently sticky since sand and other sedimentary material in the vicinity adheres to the discharged threads, thus becoming incorporated in the outer matrix of the new tube. Their adhesive properties probably tend to bond

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FIGURE 1 Photograph of *Ceriantheopsis americanus* in process of secreting new tube. Animal is about 15 cm long.

FIGURE 2. Photomicrograph of newly formed tube material removed from the column of *C. americanus*. Note everting ptychocyst among the capsules and threads of previously fired ptychocysts. Scale bar is 20 μm.

FIGURE 3. Photomicrograph of discharged ptychocyst capsule and thread. Scale bar is 20 μm.

FIGURE 4. Photomicrograph of everting ptychocyst. Note the arrangement of the uneverted thread within the capsule. Scale bar is 20 μm.

FIGURE 5. SEM of interface between column (left) and region of new tube formation (right). Scale bar is 200 μm.

FIGURE 6. SEM of newly everted ptychocyst threads forming felted meshwork of new tube. Note longitudinal ridges on the threads. Scale bar is 20 μm.

FIGURE 7. TEM, primarily of cross-sections of undischarged ptychocyst threads with 6 pleats. The strongly curved thread is a partial longitudinal section. Scale bar is 1 μm.

FIGURE 8. TEM cross-section of undischarged ptychocyst threads with 7 pleats. Scale bar is 0.5 μm.

FIGURE 9. TEM cross-section of undischarged ptychocyst thread with 10 pleats. Scale bar is 0.5 μm.

FIGURE 10. TEM longitudinal section of undischarged ptychocyst threads, probably with 6 pleats. Scale bar is 1 μm.

the everted threads to each other as well, again aiding in strengthening the tube as it forms.

The undischarged and partially discharged threads of ptychocysts stain with both basic (methylene blue, toluidine blue)and acid dyes (acid fuchsin). Since nematocysts have been classically considered to be basophilic in nature and spirocysts acidophilic (Hyman, 1940), the fact that ptychocysts are both basophilic and acidophilic may suggest some chemical differences between them and the other cnidae described to date. However, the nonspecificity of such stains in the case of many coelenterate cnidae indicates that more critical tests need to be made before much can be said concerning possible chemical differences.

Examination with SEM of the column surface of *C. americanus* in the region of new tube formation clearly shows the mass of newly fired ptychocyst threads forming the tube (Fig. 5). An enlarged view of these threads reveals that they lack any form of spination or tubules and instead have longitudinal ridges or striations running along their length, a feature previously unknown for coelenterate cnidae (Fig. 6). These ridges, representing the folds by which the thread is compressed in diameter prior to discharge, can also be seen with the phase contrast microscope, but have been heretofore undescribed.

Ultrastructure of the undischarged thread

TEM examination of the threads of undischarged ptychocysts revealed that they have a previously undescribed method of folding. All undischarged nematocyst and spirocyst threads examined to date with TEM are helically folded with multiple pleats in length, but only three in circumference. The undischarged ptychocyst thread, on the other hand, is folded accordion-like in circumference into a series of stacked pleats of varying number (Figs. 7, 8, 9). Following the terminology of Skaer and Picken (1965), the term "fold" is used to refer to the region where the surface of the thread is in fact folded, while "pleat" refers to the two thickness of the thread brought together by folding.

Due to the great variability in folding, the various types of ptychocyst threads have been categorized based on the number of pleats they contain. In the case of undischarged threads which are all infolded towards a common medial axis, the number of pleats occurring along both sides of this axis must be counted. Figure 7, for example, shows cross-sections of a symmetrically folded thread which has formed 6 pleats. Figure 8 shows an asymmetrically folded thread forming a total of 7 pleats, and Figure 9 shows a symmetrically folded thread with 10 pleats.

Another unique feature of the undischarged ptychocysts thread relates to its lack of pleating in length (Fig. 10). The threads of all spirocysts and nematocysts examined with TEM are complexly pleated, accordion-like, in length. When these cnidae discharge, the pleats become smoothed out allowing a significant increase in length of the everted thread to form a hollow cylinder (Skaer and Picken, 1965; Mariscal, 1974c). The ptychocyst thread, however, is not pleated in length and thus the length of the undischarged thread within the capsule should equal that of the fully everted thread, although it has not been possible to measure this accurately (Fig. 10).

This lack of pleating in length of the undischarged thread suggests that the ptychocyst thread may be arranged within the capsule differently than are spirocysts and nematocysts and preliminary observations suggest this to be the case. The undischarged threads of spirocysts and many nematocysts are spirally coiled around the circumference of the capsule and surrounding the central shaft region, when present. The thread of the ptychocyst, on the other hand, is arranged in the longitudinal plane of the capsule in a manner very reminiscent of the way a fire hose or ship's line is laid down to permit easy running without entanglement (Figs. 2, 4).

Ultrastructure of the capsule tip

Neither an operculum nor apical flaps is present on the capsule tip, at least in the form that these structures have been previously described for hydrozoan and anthozoan nematocysts, respectively (Fig. 11). Instead, the apical tip of the capsule appears to be folded together and sealed along several suture planes, perhaps three, although the number remains to be determined accurately. Figure 11 shows two of the triangular apical folds and one of the ruptured suture planes between them.

Ultrastructure of the discharged thread

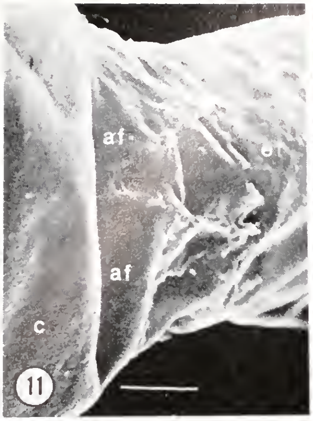
SEM of the discharged ptychocyst thread clearly shows the remarkable longitudinal ridges along which the thread is folded and compressed in diameter prior to discharge (Figs. 6, 12–13). The relief of these ridges permits the reconstruction of the method of thread folding prior to discharge, as well as allowing a tentative count of the number of pleats present (Fig. 13).

TEM of newly formed tube material provided numerous cross-sections of ptychocyst threads. Nearly all of the sectioned threads were collapsed to some degree so that the lumen was often indistinct (Figs. 14–18). In some cases, one portion of the thread would be partially inflated and another portion collapsed (Figs. 17, 18). Many of the threads had a small star or double star configuration, either collapsed (Figs. 15, 16) or partially expanded (Fig. 19).

Construction of paper models of ptychocyst threads allowed a comparison of the different patterns of pleating before, during and after discharge. Figure 20 diagrammatically shows a thread with 6 pleats both before and after discharge. Although the tip of a pleat on an uneverted thread represents a fold between the base of two pleats on the discharged thread (arrows), the same number of pleats occurred in both the undischarged and discharged state and were of equal width at any particular point on the thread (Fig. 20).

One of the most perplexing initial findings of the present study concerned the great variability in pleating in circumference of the thread, both undischarged and discharged. All undischarged nematocyst and spirocyst threads examined to date have been helically folded in essentially similar fashion and contain only three major pleats in circumference. However, from 5 to a maximum of 11 pleats (Fig. 21) have been identified in the material examined in the present study, and it is predicted that a greater range in pleating in circumference will be found for this cnida, perhaps 3 or 4 to 12.

Our preliminary measurements from a variety of preparations suggests that the widths of individual pleats may be relatively consistent. In the case of two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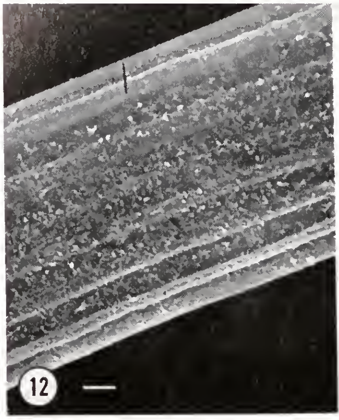
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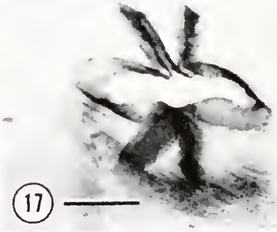
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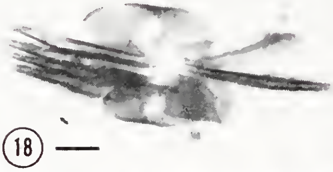
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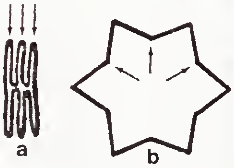
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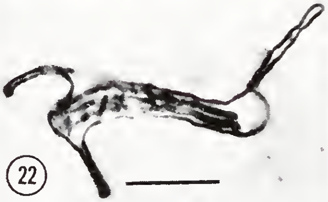
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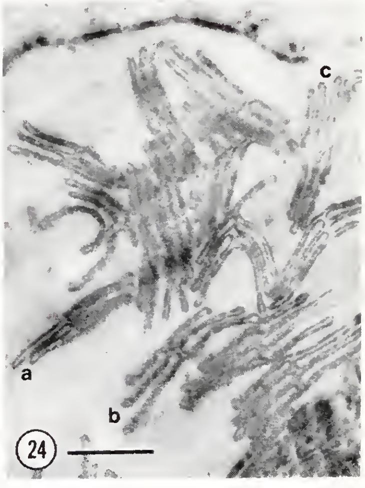
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different threads, each containing 11 pleats, it was found that the width of the everted pleats in a SEM preparation (Fig. 12) was the same as that observed in a TEM preparation (Fig. 21). In both, the mean width of a single pleat was about 0.85 μm, this being nearly identical to the mean width of pleats from all preparations measured in the present study.

Cross-sections through everting threads were especially useful in such measurements and for comparative purposes. For example, Figure 22 shows a cross-section through an everting thread containing 5 pleats, while Figure 23 shows an everting thread with 9 pleats. Note in Figure 23 that there are clearly 9 pleats on both the uneverted and everted sections at that particular point along the thread's length.

Although it was first assumed that the number of pleats would at least be consistent within a single capsule, TEM sections have revealed that a variable number of pleats can occur on a single thread. For example, Figure 24 shows an undischarged thread with 5, 6, and 7 pleats.

The consistent width of each pleat and the variable number occurring on a single thread has suggested how a nonhelically folded thread can taper in the same fashion as a helically pleated thread. If in fact the pleat width is relatively constant regardless of the number of pleats present, then a simple reduction in the number of pleats from the base of the thread to its tip could account for the

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FIGURE 11. SEM of opercular region of discharged ptychocyst capsule (c) showing apical folds (af) and ruptured suture plane between them through which the everting thread (et) passes. Scale bar is 1 μm.

FIGURE 12. SEM of everted ptychocyst thread, probably with 11 pleats, showing the pronounced longitudinal ridges by which the thread is folded in diameter prior to discharge. The fine granules may represent adhesive material. Scale bar is 1 μm.

FIGURE 13. SEM of surface of everted ptychocyst thread, probably with 8 pleats, showing relief and folding pattern of pleats. Scale bar is 1 μm.

FIGURE 14. TEM cross-section of collapsed, everted ptychocyst thread, probably with 7 pleats. Scale bar is 1 μm.

FIGURE 15. TEM cross-section of collapsed, everted ptychocyst thread with 10 pleats. Scale bar is 1 μm.

FIGURE 16. TEM cross-section of collapsed, everted ptychocyst thread, probably with 9 pleats. Scale bar is 1 μm.

FIGURE 17. TEM cross-section of everted, partially collapsed ptychocyst thread with 6 pleats. Scale bar is 1 μm.

FIGURE 18. TEM cross-section of everted, partially collapsed ptychocyst thread, probably with 8 pleats. Scale bar is 1 μm.

FIGURE 19. TEM cross-section of everted, partially expanded ptychocyst thread with 10 pleats. Scale bar is 1 μm.

FIGURE 20. Diagram illustrating eversion of ptychocyst thread: (a) cross-section of undischarged ptychocyst thread with 6 pleats; (b) cross-section of expanded ptychocyst thread with 6 pleats following eversion. The arrows point to the same folds, both before and after discharge.

FIGURE 21. TEM cross-section of everted, collapsed ptychocyst thread with 11 pleats. Scale bar is 0.5 μm.

FIGURE 22. TEM cross-section of ptychocyst thread, probably with 5 pleats, in the process of everting. Scale bar is 1 μm.

FIGURE 23. TEM cross-section of ptychocyst thread with 9 pleats in the process of everting. Note the same number of pleats on both the uneverted and everted portions of the same thread. Scale bar is 1 μm.

FIGURE 24. TEM of cross-sections of ptychocyst threads with variable number of pleats within the same capsule: (a) thread with what appears to be 5 pleats; (b) thread with 6 pleats; and (c) thread with 7 pleats. Scale bar is 0.5 μm.

observed tapering of the thread. Thus, in the case of the largest ptychocysts observed (about 75 μm capsule length in the present study), perhaps a full range of pleating occurs, whereas in the smaller ptychocysts perhaps a reduced range of pleating will be found. However, more extensive measurements will be necessary in order to verify this.

The ptychocyst thread is totally devoid of spines and, as such may represent one of the few cases of a truly atrichous cnida (Figs. 12, 13). Although no regular pattern of anything which could be interpreted as spination is present, light microscopy and SEM of everted ptychocyst threads often show a variety of fine granular material adhering to the thread surface. Since the everted threads are very sticky, it is possible that these granules represent the adhesive material (Figs. 12, 13), although at present the possibility cannot be ruled out that the granules are artifactual in nature.

The general features of the ptychocyst capsule and thread and mechanism of discharge are summarized in Figure 25.

It should be mentioned here that although the ptychocyst possesses a unique method of folding, the spirocysts and nematocysts (including holotrichs and microbasic mastigophores) also present on the column of *C. americanus* had the typical tripartite, helical folding of the thread in circumference and the characteristic accordion-like pleating of the thread in length similar to the cnidae from other species of anthozoans.

Discussion

Although it has been known for many years that ceriantheids incorporated discharged cnidae into the construction of the tubes which surround them, the identification and relative importance of these cnidae has remained unclear. It has been determined in the present study that the cnida involved in tube construction by *Ceriantheopsis americanus* represents a major new category, the ptychocyst, and that it is primarily, if not entirely, responsible for the strength and formation of the tube. The "stickiness" and interweaving capability of the long ptychocyst thread forms a tube of extreme toughness and resiliency, as well as allowing for the incorporation of sand and other sedimentary material into the outer matrix of the tube. The layered construction of the *C. americanus* tube suggests that ptychocysts are periodically added to the inside of the tube during the life of the animal. Exactly how the tube can increase in diameter to accommodate the growth of the animal remains unclear.

Some workers have indicated that spirocysts contributed to tube construction in cerianthids (*e.g.*, Robson, 1973). The present study found that spirocysts are indeed present in the newly secreted tube material of *C. americanus* but are rare compared to the tremendous numbers of ptychocysts present. Similarly, both large holotrichous isorhizas and smaller microbasic mastigophores are present in the newly secreted tube, but light and electron microscopy reveals that these nematocysts, like the spirocysts, are relatively rare. It is therefore concluded that the role of these cnidae in tube formation is negligible compared to that of the ptychocyst, at least in the case of *C. americanus*.

Aside from their function in cerianthid tube construction, the ptychocysts are completely different from all other nematocysts and spirocysts described in regard

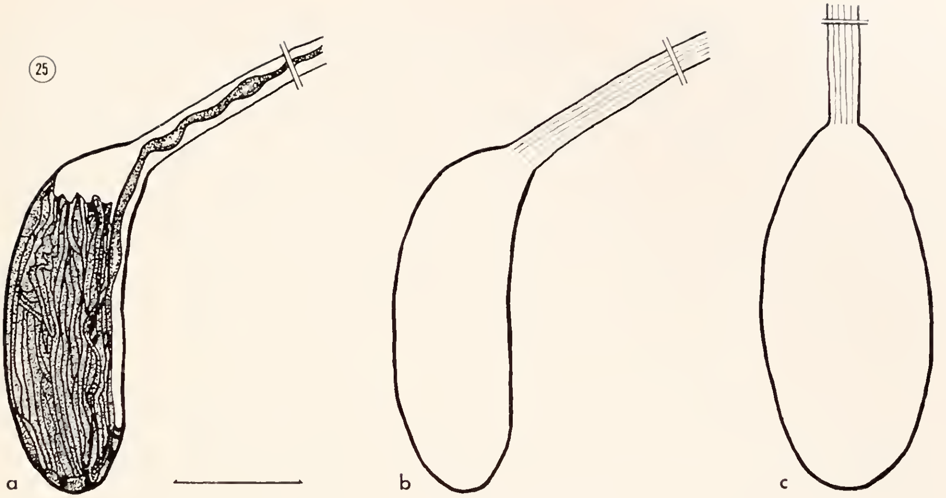

FIGURE 25. Diagram illustrating general characteristics of the ptychocyst capsule and thread: (a) side view of ptychocyst in the process of everting during discharge (note method of unfolding of everting thread); (b) side view of fully discharged ptychocyst showing longitudinal ridges on the thread representing the folds by which the thread is compressed in diameter prior to discharge; (c) frontal view of approximately the same size ptychocyst capsule as shown in (b). Scale bar is 22 μm.

to the method of folding of the thread, both before and after discharge. All spirocysts and nematocysts examined to date with the electron microscope possess a helical, tripartite pleating of the undischarged thread in circumference and an accordion-like pleating of the thread in length (*e.g.*, Mariscal, 1974c; Mariscal, Bigger and McLean, 1976; Mariscal and McLean, 1976). The ptychocyst thread, on the other hand, is not helically folded and is therefore not compressed and pleated at all in length. This points out several important differences in the functional significance and evolution of coelenterate cnidae.

In the case of a helically pleated thread, there is a significant reduction in the length and diameter of the undischarged thread which allows for it to be packed into a relatively small capsule. Helical folding thus allows for the expansion in three dimensions of an everting spirocyst or nematocyst thread, as well as imparting a rotary motion to it, somewhat analogous to the motion imparted to a bullet by the rifling of a gun barrel (Skaer and Picken, 1965).

On the other hand, nonhelical folding, characteristic of the ptychocyst, allows for an expansion of the thread in only two dimensions since there is no pleating in length. Thus the discharge of a ptychocyst thread involves a flattened belt of material (the uneverted thread) passing up through a hollow tube. By the very nature of its folding, one would not expect a strong rotary or boring motion to be imparted to the ptychocyst thread during discharge. However, the thread does appear to twist during eversion and there may be a slight rotary component associated with ptychocyst discharge as well.

With regard to classification of ptychocysts within the classical framework of coelenterate cnidae, many more questions than answers have been raised by the present study. According to the most widely used classification scheme, that of Weill (1929, 1930, 1934) as modified by Werner (1965) and Mariscal (1971, 1974c), there are two major categories of coelenterate cnidae: nematocysts and spirocysts. Based on differences in their chemical properties and structure, recent workers have generally agreed with Weill's separation of spirocysts from nematocysts proper (*e.g.*, Westfall, 1965; Schmidt, 1969, 1974; Mariscal, 1974c; Mariscal and McLean, 1976). In spite of their well known differences, however, both spirocyst and nematocyst threads have identical methods of folding and pleating within the capsule in the undischarged state. If, as is generally acknowledged, spirocysts and nematocysts are distinct enough to be placed in separate, major categories, then it seems clear that the ptychocyst represents a third major category of coelenterate cnidae in both Weill's (1934) and Schmidt's (1974) classification schemes.

Several other possibilities concerning the classification of the ptychocyst have also been considered in the present study. For example, based on light microscopical observations, the ptychocyst thread tip appears to be closed, suggesting possible affinities with nematocysts belonging to the Astomocnidae. However, the ultrastructure and method of folding of the undischarged thread has not been examined in detail in astomocnid nematocysts. Only one astomocnid nematocyst, the desmoneme, has been examined to date with TEM (Chapman and Tilney, 1959) and SEM (Mills and Mariscal, unpublished). It appears from these studies that the method of folding of desmonemes is not the same as for ptychocysts. Furthermore, all astomocnid nematocysts have been described to date only from hydrozoans. The fact that the ptychocyst occurs only in anthozoans (so far as is known) might be further evidence suggesting caution in placing the ptychocyst in the Astomocnidae until more is known about the ultrastructure of this group of nematocysts.

Another possibility is that the ptychocyst is a kind of atrichous isorhiza based on the complete absence of spination on the thread. This cnida has in fact been called an atrich by Schmidt (1972, 1974) and Carlgren (1940). Atrichs, however, have been classically considered to belong to the Stomocnidae which have threads open at the tip. The observation that the ptychocyst thread is closed at the tip would argue against ptychocysts being considered as a new type of atrich. In addition, all atrichs examined to date with the electron microscope have been found to possess spines along the length of the thread, and thus are actually holotrichous (Westfall, 1965; Schmidt, 1969, 1974; Bigger, 1976). It is therefore questionable if a truly atrichous stomocnid nematocyst exists. If in fact all previously described atrichous isorhizas are holotrichous, then the ultrastructure and method of folding of holotrichous isorhizas might profitably be examined and compared with that of ptychocysts.

Fortunately there have been several detailed examinations by TEM of the ultrastructure of the undischarged holotrich thread and it is clear that it is helically folded and pleated (Skaer and Picken, 1965; Mariscal, 1974c and unpublished). Although relatively rare, holotrichs also occur on the column of *C. americanus*, and they too are helically folded (Mariscal and Bigger, unpublished). Therefore, there seems to be no close affinities between the structure of ptychocysts and that of atrichous isorhizas or holotrichous isorhizas as classically described.

Studies are currently underway concerning the nature of the possible sensory structures associated with ptychocysts. Preliminary examination of the column of *C. americanus* with both TEM and SEM suggests that a single cilium surrounded by a circlet of shorter stereocilia is closely associated with the tip of undischarged ptychocysts. Multiciliated cells appear to surround the ptychocyte although their relationship to the ptychocyte and the discharge process, if any, is not yet clear.

To summarize the systematic status of the ptychocyst in relation to both nematocysts and spirocysts, the following revision in the classification of coelenterate cnidae by Mariscal (1974c) is proposed.

I. HELICOPTYCHONEMES—undischarged thread helically folded to form multiple pleats in length and three pleats in circumference
  A. Nematocysts—thread spined or unspined but without hollow tubules
    1. Astomocnidae—thread closed at the tip
    2. Stomocnidae—thread open at the tip
  B. Spirocysts—thread lacking spines but with hollow tubules
II. HETEROPTYCHONEMES—undischarged thread not helically folded, with a variable number of pleats in circumference but none in length
  A. Ptychocysts—thread lacking both spines and hollow tubules but with longitudinal ridges along its length

Although the ptychocyst is the first major new category of coelenterate cnida to be discovered since the spirocyst was recognized and named by Bedot in 1890, it is entirely possible that additional major new categories of cnidae remain to be recognized, perhaps among the Astomocnidae. Therefore, the above proposed revision in the classification of coelenterate cnidae has been set up to allow for the addition of new categories or sub-categories as they might be discovered. It is predicted that the threads of all toxin delivering nematocysts, presently belonging to the Stomocnidae, will turn out to be helically folded and pleated, but that some re-classification of the Astomocnidae may be necessary once these latter cnidae are examined in detail with the electron microscope.

The completely unexpected and unusual method of folding and pleating of the ptychocyst thread emphasizes the need for further study of the structure, function, mechanism of discharge, toxicology and biochemistry of the unique intracellular organelles of coelenterates which are probably among the most complex known in the animal kingdom.

Thanks are due Dr. Cadet Hand for reading a preliminary draft of the manuscript and offering valuable suggestions. Acknowledgment is also made to the Institute of Molecular Biophysics of Florida State University for the use of TEM facilities, to the Electron Microscope Laboratory of Florida State University for the use of SEM facilities and to Bill Miller for expert technical assistance. This study was supported in part by NSF Grant GB–40547 to the senior author and by the Psychobiology Program at Florida State University.

SUMMARY

Light, transmission electron and scanning electron microscopy of a cerianthid anemone reveal that the protective tube with which the animal surrounds itself

is composed almost entirely of discharged, interwoven cnidae of a heretofore undescribed type.

As opposed to the threads of all nematocysts and spirocysts described to date, the thread of the new cnida, to which has been given the name of ptychocyst, is not helically folded, forming a variable number of pleats in circumference (from 5 to 11 observed in the present study), and no pleats in length. The discharged threads are quite long (over 2 mm in some) and are non-isodiametric, tapering from a diameter of about 5 μm at the base to about 2 μm at the tip for an overall reduction in diameter of 2.5 times.

The everted thread has a number of fine ridges running along its length which can be seen using phase contrast microscopy and which represent the folds by which the thread is compressed in diameter. The thread tip is closed and the entire thread is unarmed, lacking both spines and hollow tubules. The undischarged and partially discharged threads are both basophilic and acidophilic. The capsules are bilaterally compressed and are often large (up to 75 μm long and 36 μm wide) with perhaps two distinct size classes.

The completely unique method of thread folding indicates that ptychocysts are significantly different from either nematocysts or spirocysts, both of which have helically folded and pleated threads. Ptychocysts have therefore been included in the classification of coelenterate cnidae as a third major category, equal in rank to both nematocysts and spirocysts.

LITERATURE CITED

Bedot, M., 1890. Observation sur les nématocystes. *Arch. Sci. Phys. Nat. Genève*, **22**: 606–608.

Bigger, C. H., 1976. The acrorhagial response in *Anthopleura krebsi:* intraspecific and interspecific recognition. Pages 127–136 in G. O. Mackie, Ed., *Coelenterate ecology and behavior.* Plenum Press, New York.

Carlgren, O., 1912. Ceriantharia. *Danish Ingolf-Exped.*, **5a(3)**: 1–78.

Carlgren, O., 1940. A contribution to the knowledge of the structure and distribution of the cnidae in the Anthozoa. *Lunds Univ. Årsskrift, N. F., Avd. 2*, **36**: 1–62.

Chapman, G. B., and L. G. Tilney, 1959. Cytological studies on the nematocysts of Hydra. I. Desmonemes, isorhizas, cnidocils and supporting structures. *J. Biophys. Biochem. Cytol.*, **5**: 69–78.

Frey, R. W., 1970. The *Lebensspuren* of some common marine invertebrates near Beaufort, North Carolina. II. Anemone burrows. *J. Paleontol.*, **44**: 308–311.

Hyman, L. H., 1940. *The invertebrates: Protozoa through Ctenophora, Vol. I.* McGraw-Hill, New York, 726 pp.

Mariscal, R. N., 1971. Effect of a disulfide reducing agent on the nematocyst capsules from some coelenterates with an illustrated key to nematocyst classification. Pages 157–168 in H. M. Lenhoff, L. Muscatine and L. V. Davis, Eds., *Experimental coelenterate biology.* University of Hawaii Press, Honolulu.

Mariscal, R. N., 1974a. Scanning electron microscopy of the sensory surface of the tentacles of sea anemones and corals. *Z. Zellforsch.*, **147**: 149–156.

Mariscal, R. N., 1974b. Scanning electron microscopy of the sensory epithelia and nematocysts of corals and a corallimorpharian sea anemone. *Proc. Second Int. Coral Reef Symp.*, **1**: 519–532.

Mariscal, R. N., 1974c. Nematocysts. Pages 129–178 in L. Muscatine and H. M. Lenhoff, Eds., *Coelenterate biology: reviews and new perspectives.* Academic Press, New York.

Mariscal, R. N., C. H. Bigger, and R. B. McLean, 1976. The form and function of cnidarian spirocysts. 1. Ultrastructure of the capsule exterior and relationship to the tentacle sensory surface. *Cell Tissue Res.*, **168**: 465–474.

Mariscal, R. N., and R. B. McLean, 1976. The form and function of cnidarian spirocysts.

2. Ultrastructure of the capsule tip and wall and mechanism of discharge. *Cell Tissue Res.*, **169**: 313–321.

McMurrich, J. P., 1890. Contributions on the morphology of the Actinozoa. I. The structure of *Cerianthus americanus*. *J. Morphol.*, **4**: 131–150.

Parducz, B., 1967. Ciliary movement and coordination in ciliates. Pages 91–128 in G. H. Bourne and J. F. Danielli, Eds., *International review of cytology*. Academic Press, New York.

Robson, E. A., 1973. The discharge of nematocysts in relation to properties of the capsule. *Publ. Seto Mar. Biol. Lab.*, **20**: 653–665.

Schmidt, H., 1969. Die Nesselkapseln der Aktinien. *Helgol. Wiss. Meeresunters.*, **19**: 284–317.

Schmidt, H., 1972. Die Nesselkapseln der Anthozoen und ihre Bedeutung fur die phylogenetische Systematik. *Helgol. Wiss. Meeresunters.*, **23**: 422–458.

Schmidt, H., 1974. On evolution in the Anthozoa. *Proc. Second Int. Coral Reef Symp.*, **1**: 533–560.

Skaer, R. J., and L. E. R. Picken, 1965. The structure of the nematocyst thread and the geometry of discharge in *Corynactis viridis* Allman. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, **250**: 131–164.

Torrey, H. B., and F. L. Kleeberger, 1909. Three species of *Cerianthus* from southern California. *Univ. Calif. Publ. Zool.*, **6**: 115–125.

Weill, R. F., 1929. New results from the study of coelenterate nematocysts. *Proc. Nat. Acad. Sci.*, **15**: 887–892.

Weill, R. F., 1930. Essai d'une classification des nématocystes des cnidaires. *Bull. Biol. France-Belg.*, **64**: 141–153.

Weill, R., 1934. Contribution a l'étude des cnidaires et de leurs nématocystes. *Trav. Stat. Zool. Wimereux*, **10–11**: 1–701.

Werner, B., 1965. Die Nesselkapseln der Cnidaria, mit besonderer Berucksichtigung der Hydroida. I. Klassification und Bedeutung fur die Systematik und Evolution. *Helgol. Wiss. Meeresunters*, **12**: 1–39.

Westfall, J. A., 1965. Nematocysts of the sea anemone *Metridium*. *Am. Zool.*, **5**: 377–393.

Reference: *Biol. Bull.*, **152**: 406–414. (June, 1977)

ISOLATION OF NEMATODE INHIBITOR FROM HEMOLYMPH OF THE SNAIL, *HELIX ASPERSA*

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Natural resistance of molluscan hosts to the invasion of parasitic trematodes and nematodes, involving both cellular and humoral responses, has been reported and reviewed extensively (Tripp, 1963, 1974; Acton, 1970). The presence of biologically active material in gastropod hemolymph which has an inhibitory effect on maturation and reproduction of a nematode symbiont has been reported recently (Ratanarat-Brockelman, 1975). This symbiotic relationship involves low infestations (2–8 third stage larvae) of *Rhabditis maupasi* in the mantle cavity of the food snail *Helix aspersa.* Mechanical expulsion of worms occurs when higher levels of infestation are made, suggesting two possible regulatory mechanisms: competition among nematodes, or host control over the level of infestation.

Although the worm was found free in the mantle cavity of the host in experimental infections, other, naturally-infected, snails have been found with worms within the visceral mass as well (G. J. Jackson, personal communication). Evidently the worms can penetrate the cavity wall, and perhaps routinely irritate or rupture the wall in anchoring and feeding, thus exposing themselves to the inhibitor. This aspect of the relationship, as well as the precise source of nutrition of the worms, needs additional clarification.

Third stage larvae that became established in the snail's mantle cavity did not undergo molting or morphological development until the snail died. The nematode larvae then began to grow, molted twice, became sexually mature and reproduced prolifically until the snail tissue was completely consumed. At this time they entered the soil provided, and were capable of reinfesting a new host.

An inhibitor which retards development and reproduction of worm populations cultivated *in vitro* was isolated from fresh snail plasma (Ratanarat-Brockelman, 1975). This inhibitor interfered with larval development and with differentiation of the reproductive organs in maturing worms. The action of the inhibitor varied directly with concentration, and it was found to have two complementary parts: a diffusable cofactor and a proteinaceous component. The present report presents further results on the analysis of the proteinaceous component.

MATERIALS AND METHODS

Nematode

Specimens of *Rhabditis maupasi* were grown axenically on agar slant cultures. The offspring had been subcultured continuously since 1972. A mixed population of the nematode was harvested from an agar culture one day before each bioassay and suspended in 5% sterile dextrose solution. Third stage larvae were separated from other stages in a neo-Baermann apparatus (Ratanarat-Brockelman and Jackson, 1974) at 20° C. The larvae which migrated out were collected in 5% dextrose solution for inoculation into test cultures.

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Snail plasma

The specimens of *Helix aspersa* used for bleeding in this report were originally obtained from a fish market in Bronx, New York. Half of each lot purchased was dissected and examined for helminthic infection. The remainder were kept in aestivation at 21° C. Before the snails were used in experiments, they were rehydrated in distilled water and allowed to feed on oatmeal.

Hemolymph was collected from 300 uninfected snails by making an incision along the *vena magna* and pipetting the hemolymph out until the bleeding stopped. After this procedure, the snails were partially shelled, making a second incision possible in the visceral sac where hemolymph was again collected and pooled to 300 ml volumes. Gelatinous materials (including free cells and the respiratory pigment, hemocyanin, accompanying hemolymph during collection) were separated from the plasma by refrigerated ultracentrifugation in a Beckman Model L preparative ultracentrifuge at 78,000 × g for 2 hr. The plasma which remained as supernatant was pooled and then concentrated by pressure filtration through Visking cellulose tubing (casing size 8DC) as described by Craig (1968); 50 ml of plasma was concentrated to 10 ml in four hr at 4° C. After concentration, the filtrate containing the co-factor was collected, sterilized by Seitz filtration and stored at 4° C for biassay (Ratanarat-Brockelman, 1975). The plasma concentrate retained in the tubing is referred to as "nondiffusable" since the molecular weights of the components were all higher than 10,000 (Craig, 1968). This concentrate was sterilized by Seitz filtration and refrigerated.

Isolation of inhibition

Separation by ion exchange chromatography. The concentrated snail plasma was dialyzed in a tube of Diaflow UM 3 membrane against cold potassium phosphate buffer of ionic strength 0.15 M and pH 7.8, and applied to a chromatographic column (4.0 × 60 cm) of DEAE-A25 Sephadex. The sample volume at each run was 15 ml and contained 60 mg of protein. It was eluted at 4° C employing an elution schedule of a linear salt gradient (King, 1968). The gradient was produced by connecting two buffer vessels. The starting buffer, connected to the column, was potassium phosphate (pH 7.8, ionic strength 0.15 M). The second vessel, connected with the first one at the bottom, was filled with limiting buffer whose salt concentration was 0.6 M NaCl. The flow rate of buffer was 40 ml per hr. Fractions of 3 ml each were collected and the protein content was determined spectrophotometrically at 280 nm. All protein-containing fractions were pooled to nine portions according to optical density and concentrated in boiled and prechilled Visking cellulose tubing (23/32 in diameter) against polyethyleneglycol, mol. wt. 6000, which reduced the volume from 60 ml to 5 ml. The concentrated fractions were dialyzed against 0.85% NaCl overnight and assayed for protein content by the method of Lowry, Rosebrough, Farr, and Randall (1951). They were sterilized by Millipore membrane filtration type Swinnex GS-13. Most procedures were carried out at 4° C.

Gel filtration. Gel filtration was done on columns of Sephadex G-200. The packed columns of 1.6 × 120 cm were washed and eluted with 0.15 M potassium phosphate buffer, pH 7.8. Void volumes, determined by passing 0.2 ml of 1% Blue Dextran (Pharmacia, Uppsala, Sweden) dissolved in the same buffer through the column, were found to be about 57 ml. These columns were to fractionate the active protein obtained from the cellulose ion exchanger.

Two ml of inhibitor (2.0 mg protein) was fractionated at a time by gel filtration in potassium phosphate buffer at a flow rate of 12 ml per hr. Individual fractions of 2.6 ml were collected and determined spectrophotometrically for protein content at 280 nm which gave a density profile with three peaks. The three protein portions were again concentrated individually to 2 ml, dialyzed in 0.85% NaCl, and sterilized by membrane filtration.

Starch zone electrophoresis. Purity of the isolated inhibitory protein was examined by starch zone electrophoresis (Kunkel and Slater, 1952; Dusanic and Lewert, 1963) in a veronal buffer of pH 7.8 with an ionic strength of 0.1 M. For each run, 2 ml of sample containing 400 μg of inhibitor was applied to a 50 × 4 × 0.7 cm starch block. The electrophoretic procedure, at 4° C and at 300 volts, was stopped after either 36, 38, or 72 hrs. Each block was partitioned into 1 cm segments. Each segment was eluted with 5 ml of veronal buffer and tested for protein content in individual aliquots (Lowry *et al.*, 1951).

Qualitative determination of the purified factor

Biochemical compounds which usually accompany protein were determined. Montgomery's test (1957) was used to examine for the presence of glycogen. Presence of nucleic acid was searched for by precipitation in 0.2 N acetic acid followed by safranin staining (Rapoport and Raderecht, 1962). The inhibitor was also placed in ether and chloroform to test its solubility, which would give a primary indication of lipid.

Bioassay technique

Protein concentrations of all fractions were quantified by the method of Lowry *et al.* (1951). Dilutions for experimental assays were adjusted with normal saline to equalize the concentration at 1 mg per ml, then mixed 1 : 1 with hemolymph diffusate. One-ml volumes of this were distributed (six replicates per protein portion) to 25-ml Delong culture flasks which contained the following components: 1 ml of basal Pfahnstiel peptone broth (Pf broth), 0.5 ml raw rabbit liver extract (RLE) containing 30 mg protein per ml, and 0.5 ml of 5% dextrose suspension of about 46 larvae (range 37–59). Thus, the final concentration of snail inhibitory protein was 166.7 μg per ml of culture medium. The control group received 1 ml of 0.85% NaCl solution in place of snail plasma protein. Culture flasks were kept in the dark while being shaken at 40 strokes per minute at 21° C to promote aeration.

Evaluation

The nematode population in each flask was counted microscopically on day 7 over a grid, and on day 14 by dilution and volumetric sampling.

Because of the inhibitor, the rates of population increase differed in the various groups of culture media, and thus they could be used as a bioassay of the activity of the inhibitor (Ratanarat-Brockelman, 1975). Population growth in all cultures during at least the first two weeks was approximately exponential. The rate of population increase during an interval of 14 days could be calculated from the equation $N_{(14)} = N_{(0)}e^{14r}$, where N is the number of worms. Differences among mean rates of increase were tested by analysis of variance.

RESULTS

Isolation by ion-exchange chromatography

A typical elution pattern of inhibitory protein is shown in the ion-exchanger profiles of Figure 1, showing nine major peaks of proteins. These nine protein fractions were bioassayed on nematode populations, each fraction with six replicates. As is illustrated in Figure 2, protein fraction 3, eluted at 0.34 M NaCl gradient (elution volume 770 to 830 ml), resulted in the lowest rate of population increase (0.151/day), which was significantly lower than that of the control cultures receiving no snail plasma protein (0.254/day) $F_{8,50} = 7.04$, $P < 0.05$ (see Table 1). Populations receiving fractions 2 and 7 also exhibited lower mean rates

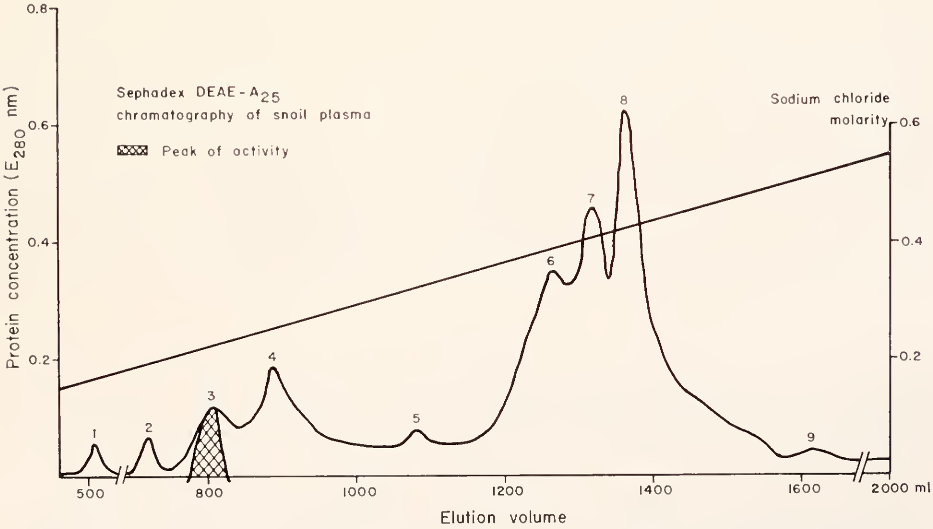

FIGURE 1. Separation of plasma of *Helix aspersa* on cellulose ion-exchanger, Sephadex DEAE-A 25. Column dimension is 4.0 × 60 cm; sample volume, 15 ml (60 mg protein); potassium phosphate buffer, 0.15 M, pH 7.8 salt gradient 0.6 M NaCl, flow rate 40 ml/hr, fraction volume 3 ml. All steps were performed at 4° C. Shaded area designates peak of activity.

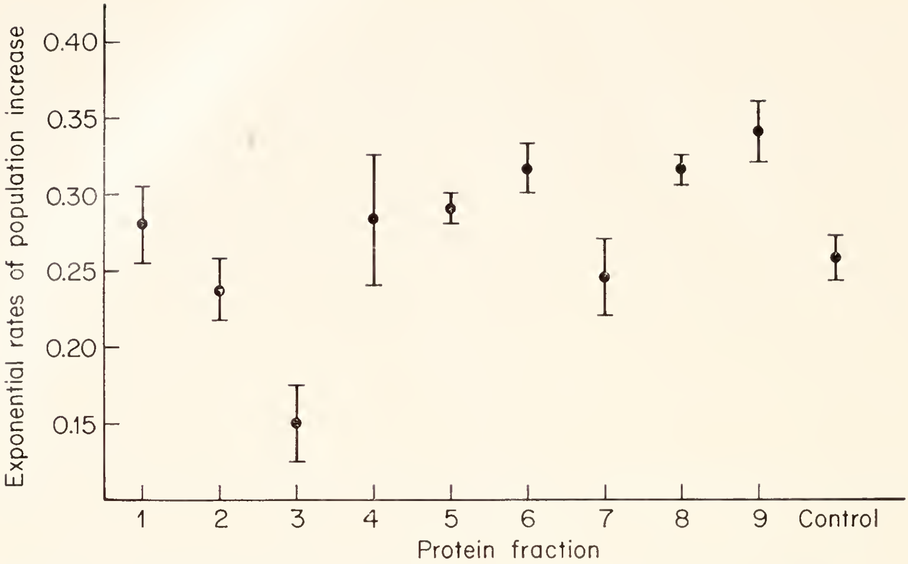

FIGURE 2. Exponential rates of population increase (r) of *Rhabditis maupasi* in nine groups of culture receiving nine different protein fractions separated by cellulose ion-exchanger, Sephadex DEAE-A 25. Time of cultivation was 14 days. Vertical lines represent standard deviations.

of increase (0.238/day and 0.245/day respectively) when compared with that of the control group, but the differences were not significant. Protein peak 3 thus contained most of the inhibitory effects.

Results from the bioassay are further illustrated in Figure 3 which shows log population sizes of nematodes against time. For clearer presentation the 60 cultures were pooled into three groups as follows. One group contained the six control cultures, which showed an average growth of population to $\log_{10}N = 3.04$ (s.d. ± 0.11) within 14 days. A second group of six cultures receiving protein fraction 3 showed suppression of population growth to $\log_{10}N = 2.23$ (s.d. ± 0.29). A third group of cultures receiving the remaining fractions, 1, 2, 4, 5, 6, 7,

TABLE I

Analysis of variance of Rhabditis maupasi *population growth rates from nine groups of cultures. Each group (six replicates) received one of the nine protein fractions obtained by fractionation of snail hemolymph protein on an ion-exchange column.*

Source of variation	d.f.	Mean square	F	*P*
Among treatments	9	188.96	4.82	<0.001
Control *vs.* nine expt. groups	1	101.78	2.59	NS
Among nine fractions	8	276.13	7.04	<0.05
Error	50	39.19		
Total	59	66.44		

8, and 9 (each with six replicates), when pooled, showed the highest mean population size of the nematodes ($\log_{10}N = 3.30$, s.d. ± 0.37).

Fractionation by gel filtration

The further step of fractionating the inhibitory protein on Sephadex G-200 yielded three more peaks eluted at 50, 78 and 150 ml, designated peak 3a, 3b, and 3c, respectively (Fig. 4). The results of bioassay are summarized in Table II, which shows that complete inhibition of reproduction occurred in the cultures receiving protein peak 3c. Although 68.2% of the initial inoculum showed sexual differentiation, in which bursae could be recognized in male worms, the reproductive organs of both sexes remained poorly developed. In male worms, the testes attained only a narrow, straight tubular shape with no coiling. The germ cells were sparse and stained poorly with 1% neutral red solution, suggesting impaired spermatocyst production. Ovaries, uteri and vulva developed in females but contained no eggs. Malfunction of the sexual organs evidently curtailed

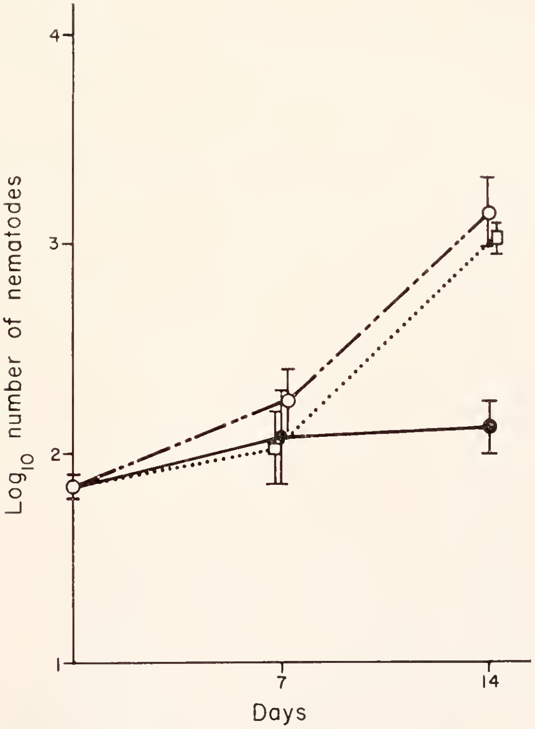

FIGURE 3. Bioassay of different fractions of hemolymph plasma on populations of *Rhabditis maupasi* grown *in vitro*. The three groups of populations shown consist of: first, means of 48 cultures receiving protein peaks, 1, 2, and 4 to 9, (open circles with broken line); secondly, cultures receiving protein peak 3 (solid circles, with full line); and thirdly, control cultures (squares, with dotted lines). Each treatment had six replicates. Vertical bars are standard deviation of log population size.

TABLE II

Rate of Rhabditis maupasi *population growth in three groups of cultures receiving separated protein fractions from Sephadex G-200. Control received only raw rabbit liver extract (four replicates).*

	Control	Cultures receiving fraction		
		3a	3b	3c
Rate of population growth (r)	0.114	0.109	0.070	0.001
Standard deviation	0.06	0.01	0.01	0*
Per cent maturity	100	100	95	68.2**

* Only two larvae were produced in one culture.

** 14.8% remained undeveloped, and 17% developed to stage 4 larvae. There were significant differences in population growth rates among fractions ($F_{2,12} = 8.34$, $P < 0.05$).

offspring production during the 14 days of cultivation, whereas two generations were born to the nematodes in control groups.

The rates of population increase in nematode cultures receiving the two other protein fractions were significantly higher than the group receiving protein peak ($F_{2,12} = 8.34$, $P < 0.05$). Thus, protein peak 3c evidently contained most of the inhibitor.

Purification of inhibitory protein

After the initial step of purification by rechromatographing the active peak 3c alone on a column of Sephadex G-200, the sample obtained was examined

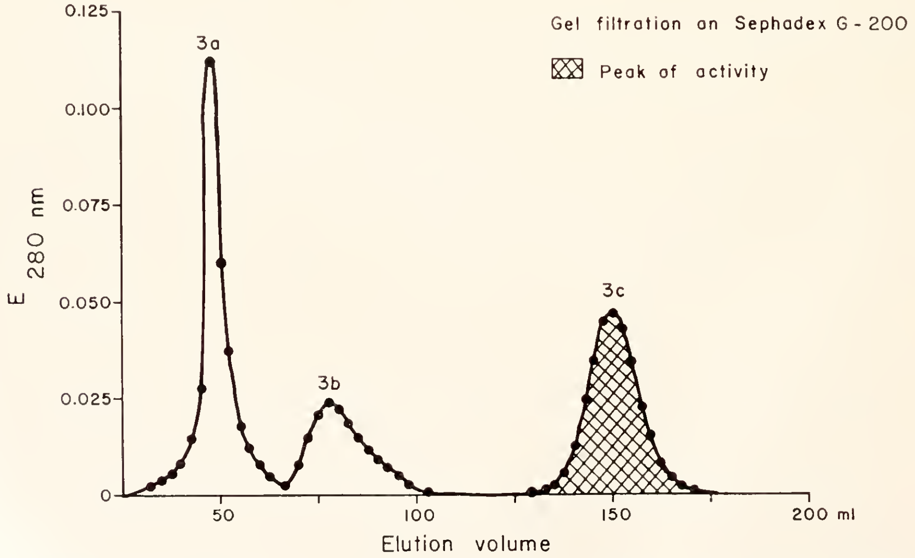

FIGURE 4. Gel filtration of active peak on a column of Sephadex G-200. Column dimension is 1.6 × 120 cm; sample size, 2 ml (2.0 mg protein); potassium phosphate buffer, 0.15 M, pH 7.8; flow rate, 12 ml/hr; fraction volume, 2.6 ml.

electrophoretically on a starch bed. Only one band appeared at each run, suggesting purity of the sample.

Determination of accompanying substances

Neither the test for the presence of glycogen nor that for nucleic acid was positive. The inhibitor did not dissolve in the fat solvents ether and chloroform, suggesting the absence of a lipid component. The inhibitor reacted with the folin-phenol reagent of Lowry *et al.* (1951) and thus appears to be protein.

DISCUSSION

The effects of a hemolymph factor occuring in *Helix aspersa* on the maturation of the nematode *Rhabditis maupasi* have been described recently by Ratanarat-Brockelman (1975). In that report, experimental results suggested a proteinaceous nature of the factor. The finding led to the present investigation, especially intended to establish whether this substance is identical to protectin, an agglutinin of glycoprotein nature which has been found to have a protective function in *Helix pomatia* (Prokop, Uhlenbruck, Rothe, and Cohen, 1974). However, the isolated and purified hemolymph factor reported here was characterized as to protein, not glycoprotein, as the Montgomery test (Montgomery, 1957) for glycogen gave a negative result. Further characterization of the inhibitor will have to await more extensive biochemical work.

The *in vitro* cultivation experiments, using growth media alone and in combination with the inhibitor, illustrated that the inhibitor suppressed the normal function of the reproductive organs but not growth. Although reproductive organs developed in some of the worms, fertilization and reproduction scarcely occurred even in the presence of ample nutrients. The purified factor allowed only 68.2% of the larvae to become sexually mature, although the protein concentration used was only 166 g/ml medium, compared to the much higher concentration required (at least 10 mg) when crude snail extract was used (Ratanarat-Brockelman, 1975). The effect of the inhibitor is not to destroy the nematodes entirely; instead, it permits a commensal relationship between the nematode larvae and the molluscan host with minimal (if any) damage to the host, and helps regulate the population level of the worm.

A full understanding of the significance of this inhibition must include a probable or at least plausible evolutionary explanation of the relationship. I know of no field work on natural populations of these species, but field observations of other *Rhabditis* species in other pulmonates yield a general life history consistent with my laboratory observations (see Filipjev and Schuurmans Stekhoven, 1941; Mengert, 1953; Ratanarat-Brockelman and Jackson, 1974). A reasonable hypothesis is that the inhibitor evolved as a host mechanism to help prevent internal infection of the snail. The worms then evolved a capability of living mostly or completely outside the host tissue and reacting to the disappearance of the inhibitor at the host's death as a cue to resume development and reproduce. It seems unlikely that the nematode could not evolve a chemical counter-defense against such a low concentration of inhibitor if there were selective pressure to do so, if this were the snail's only available defense. The commensal worm thus still gains a large ready food supply at the death of the snail, a means of dispersal, and a relatively stable moist environment.

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SUMMARY

Hemolymph plasma of the snail *Helix aspersa* which inhibits maturation and reproduction of its mantle cavity-inhabiting nematode, *Rhabditis maupasi,* was separated biochemically for the active proteinaceous component.

Isolation of the active inhibitor was performed using ion-exchange chromatography in combination with subsequent gel filtration. The isolated peaks were bioassayed *in vitro* on nematode larvae. The fractions harboring inhibitory protein suppressed larval growth and adult reproduction *in vitro.*

The isolated fraction was purified by gel filtration and characterized on the basis of a single band on starch zone electrophoresis and positive reaction only with folin-phenol reagent.

LITERATURE CITED

ACTON, R. T., 1970. *Immunobiological and immunochemical studies of the oyster Crassostrea virginica.* Manuscripts Information Corp., New York, 134 pp.

CRAIG, L. C., 1968. Ultrafiltration. Pages 129–131 in C. Williams Jr., and M. W. Chase, Eds., *Methods in immunology and immunochemistry, Vol. II.* Academic Press, New York.

DUSANIC, D. E., AND R. M. LEWERT, 1963. Alterations of proteins and free amino acids of *Australorbis glabratus* after exposure to *Schistosoma mansoni* miracidia. *J. Infect. Dis.,* **122**: 243–247.

FILIPJEV, I. N., AND H. H. SCHUURMANS STEKHOVEN, JR., 1941. *A manual of agricultural helminthology.* E. J. Brill, Leiden.

KING, T. P., 1968. Cellulose ion exchange chromatography. Pages 154–162, in C. Williams Jr., and M. W. Chase, Eds., *Methods in immunology and immunochemistry, Vol. II.* Academic Press, New York.

KUNKEL, H. G., AND R. J. SLATER, 1952. Zone electrophoresis in a starch supporting medium. *Proc. Soc. Exp. Biol. Med.,* **80**: 42–46.

LOWRY, H. H., N. J. ROSEBROUGH, H. L. FARR, AND R. J. RANDALL, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

MENGERT, H., 1953. Nematoden and Schnecken. *Z. Morphol. Tiere,* **41**: 311–349.

MONTGOMERY, R., 1957. Determination of glycogen. *Arch. Biochem. Biophys.,* **67**: 378–386.

PROKOP, O., G. UHLENBRUCK, A. ROTHE, AND E. COHEN, 1974. Protectins: past, present problems and perspectives. *Ann. N. Y. Acad. Sci.,* **234**: 228–231.

RAPOPORT, S. M., AND H. J. RADERECHT, 1962. Nachweis der Nukleinsäuren. Page 133–135 in *Physiologisch chemisches Praktikum.* VEB Verlag Volk und Gesundheit, Berlin.

RATANARAT-BROCKELMAN, C., 1975. Inhibition of *Rhabditis maupasi* (Rhabditidae; Nematoda) maturation and reproduction by factors from the snail host, *Helix aspersa. J. Invert. Pathol.,* **25**: 229–237.

RATANARAT-BROCKELMAN, C., AND G. J. JACKSON, 1974. *Rhabditis maupasi:* occurrence in food snails and cultivation. *Exp. Parasitol.,* **36**: 114–122.

TRIPP, M. R., 1963. Cellular responses of mollusks. *Ann. N. Y. Acad. Sci.,* **113**: 467–474.

TRIPP, M. R., 1974. Molluscan immunity. *Ann. N. Y. Acad. Sci.,* **234**: 23–27.

Reference: *Biol. Bull.*, **152**: 415–423. (June, 1977)

THE CIRCULATING HEMOCYTE POPULATION OF THE MOLE-CRAB *EMERITA* (= *HIPPA*) *ASIATICA* MILNE EDWARDS

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The circulating hemocyte populations of crustaceans differ from species to species (Yeager and Tauber, 1935). The magnitude of interspecific variation in the hemocyte population of crustaceans appears to be very wide, ranging from 286 cells/mm^3 (*Astacus fluviatilis*, Hardy, 1892) to 128,000 cells/mm^3 (*Callinectes sapidus*, Sawyer, Cox and Higginbottom, 1970). In part, such variations could be due to diluents used to prevent clotting of the hemolymph and clumping of the cells (Stewart, Cornick and Dingle, 1967). Yeager and Tauber (1935) also reported intraspecific variation in hemocyte populations of crustaceans. The intraspecific variations in hemocyte populations cannot be entirely explained by the differences in techniques used in the counting procedures. In *Eriocheir sinensis*, Bauchau and Plaquet (1973) have shown variations in total hemocyte count in relation to different physiological conditions of the animal. In addition, environmental factors may also influence the hemocyte population in crustaceans. Dean and Vernberg (1966) have shown that total hemocyte counts in *Uca pugilator* acclimated to 30° C are higher than those of crabs acclimated to 10° C. Although these studies suggest that variations in the circulating hemocyte population may result from a number of variables including techniques, physiological factors and environment, the mechanism underlying such variations has not hitherto been elucidated.

The purpose of the present investigation is to evaluate the obvious factors which may influence circulating populations of hemocytes of the mole-crab *Emerita asiatica*. This would provide, (first), a firm basis for the determination of normal hemocyte counts in crustaceans generally, as well as for future experimental investigations on the animal; and (secondly), elucidation of the mechanism underlying the maintenance of hematological equilibrium in crustaceans.

Materials and Methods

Specimens of the mole-crab *Emerita asiatica* were collected from the sandy shores of Madras beach, opposite to University buildings. Collections were made twice a day, between 8 AM and 1 PM and between 2 PM and 3 PM. Animals were used for analysis within six hours. In the laboratory, animals were kept in rectangular glass troughs containing sand and sea water obtained from the area of collection. Only female crabs in the intermolt stage were used in this study. Prior to every analysis, crabs were immersed in filtered sea water at room temperature (28–30° C) for few minutes. The carapace length was measured, and the water content of the hemolymph was determined. Blood samples were collected by cutting the first walking leg on one side of the animal. Care was taken to avoid mixing fine sand grains or sea water with the blood sample. Total hemocyte counts (THC) were made in a Neubauer (improved double) hemocytometer. Care was taken not to touch the chamber or coverslip, as it is known that fingerprints may also affect the counts (Dacie and Lewis, 1968). The coverslip was placed horizontally on the chambers and pressed gently on the sides. The hemolymph was allowed to fill the chamber in capillary motion about 5 sec after cutting the leg. The second chamber was filled subsequently after filling the first chamber.

In most of the previous investigations (except Drach, 1939 and Hoffmann, 1969), the hemolymph was diluted either with anticoagulants or with antiagglutinants (or with saline or Ringer's solutions containing one of the above), in order to avoid gelification of plasma and agglutination of cells. In this study neither of these diluents were used. It was reported earlier (Ravindranath, 1975a) that the gelification of plasma in *Emerita asiatica* occurs at room temperature 150 sec after amputating the leg. It may be noted that within about 5 sec of amputation of the appendage, the hemolymph fills the chamber and the cells settle within 60 to 100 sec. Plasma gelification which may occur subsequently does not alter the position in which cells have settled. However, as reported earlier (Ravindranath, 1975a), alteration of cells occur after about 5 min under normal conditions at 30° C. This does not interfere with counting as the cells are clearly visible even after alteration. Agglutination of cells in the hemocytometer was observed only when total count exceeded 20,000 cells/mm^3, which is a very rare condition in normal, agile and healthy animals. Counts were made in all squares of both upper and lower chambers of the hemocytometer. The hemocyte count/mm^3 of hemolymph was calculated from each hemocytometer count and recorded separately.

For studying the effect of size on the cell population, counts were taken in normal, intermolt animals belonging to different size groups from 22 mm to 34 mm. The effect of the time of day was studied on six normal animals for each hour during different days. The counts for 9 o'clock analysis were taken between 9:00 AM and 9:45 AM; similarly, counts were taken for different hours up to 6 o'clock in the evening. The effect of thermal stress was studied following an earlier report (Ravindranath, 1975a), by subjecting the animals to thermal shock for ten minutes. This was achieved by immersing the animal in sea water maintained at required temperature. All the observations were made betwen May and August, 1975. The statistical analyses carried out in the present study include Student's *t*-test and analysis of variance.

RESULTS

General characters

The granular hemocytes, together with their modified versions such as plasmatocytes and cystocytes (Ravindranath, 1975b), constitute more than 95% of the hemocyte population. In fact, the total hemocyte count (THC) represents primarily the granular hemocytes, as other cell types such as spherule cells and adipohaemocytes are very rare. Spherule cells, dividing granular hemocytes, binucleate and asymmetically dividing granular cells are frequently observed in the afternoon analyses of the hemolymph and also in the hemolymph of postmolt and injured intermolt animals.

TABLE I

Total counts and distribution of hemocytes of Emerita asiatica *in the squares of the hemocytometer.*

Blood Drop	Upper Corner		Central	Lower		THC mm³	Difference between first and subsequent drops	Difference between highest and lowest number of cell among five squares
	1 mm²	2 mm²	mm²	1 mm²	2 mm²			
First	715 (21.14)	661 (19.8)	724 (21.69)	619 (18.54)	619 (18.54)	6,676	338	105 (3.15)
Subsequent	617 (19.78)	698 (22.02)	636 (20.06)	611 (19.28)	607 (19.15)	6,338		91 (2.87)
First	1,184 (21.35)	1,104 (19.89)	1,135 (20.46)	1,058 (19.06)	1,064 (19.18)	11,090	2,056	126 (2.29)
Subsequent	976 (21.06)	934 (20.67)	905 (20.03)	902 (19.97)	800 (17.71)	9,034		176 (3.89)
First	886 (21.89)	819 (20.24)	846 (20.90)	745 (18.41)	747 (18.46)	8,086	374	141 (3.48)
Subsequent	790 (20.45)	833 (21.60)	749 (19.42)	742 (19.24)	742 (19.24)	7,712		91 (2.36)
First	925 (21.16)	977 (22.35)	880 (20.15)	801 (18.32)	788 (18.02)	8,742	748	189 (4.33)
Subsequent	882 (22.06)	791 (19.89)	830 (20.76)	762 (19.05)	732 (18.31)	7,994		150 (3.75)
First	900 (21.40)	894 (21.20)	849 (20.20)	794 (20.20)	765 (18.88)	8,404	1,400	135 (3.18)
Subsequent	761 (21.73)	770 (22.27)	709 (20.25)	652 (18.62)	610 (17.70)	7,004		160 (5.03)

(Numbers in parenthesis are percentage values.)

Distribution of hemocytes in the hemocytometer

Table I shows the patern of distribution of hemocytes in the squares of the chamber, when the chambers are filled in single capillary action with fresh, undiluted hemolymph. The number of cells present in the squares are not uniform. In the corner squares that are toward the central ridge, there are always more cells than in squares toward the side of entry of blood. Although the numbers in different squares are unequal, the central square always contained about 20% of the total cell distributed in five squares. Statistically, the number of cells in the central square is not significantly different from the mean number of cells distributed in all five squares. An interesting feature pertaining to THC noticed in the present study is the difference in the number of cells present in the first drop and subsequent drops. From observations made on 41 crabs, it is noted that the first drop yielded an average count of 8185 ± 2212 cells/mm^3, which is higher than the number obtained from the second drop of hemolymph (7451 ± 2410 cells/mm^3). The cause for such differences between first and subsequent drops has been suggested to be due to adhesion of cells to the wounded sites (Wigglesworth, 1956; Matsumoto and Sakurai, 1956). Size and time of day do not influence in any way the difference in the number of cells between first and subsequent drops.

THC in mature females

The magnitude of hemocyte counts in normal mature females show a fairly wide range. The THC values presented in this study are the average of the values of first and subsequent drops. The THC varies from 3723 cells to 14,185 cells/mm^3, the mean being 7891 ± 1778 cells/mm^3.

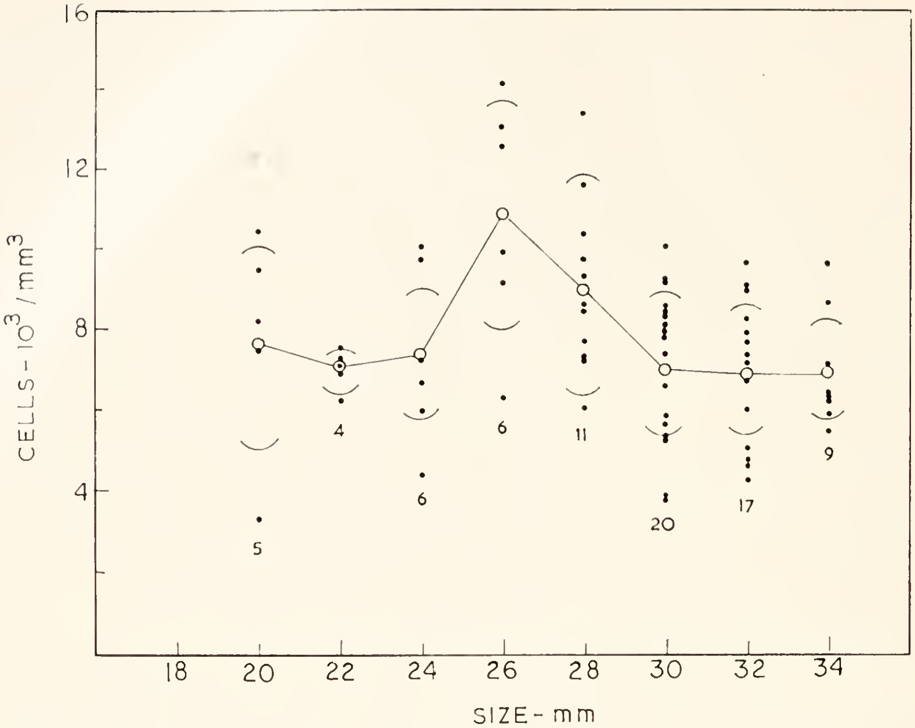

FIGURE 1. Variation in numbers of circulating hemocytes in relation to size for the mole-crab, *Emerita asiatica* (intermolt females). Mean values for each size group are shown as circles. The numbers given below the mean values are each sample size. Parentheses above and below the mean values show standard deviations.

Size as a factor influencing THC

The wide variations in THC's reported above could be due to differences in size (Fig. 1). The count is higher in animals belonging to size groups 26–28 mm than in size groups 22–24 mm, and 30–34 mm. The mean value in the size groups 30 mm, 32 mm, and 34 mm remains somewhat constant.

The variations observed in THC's in different size groups could be due to differences in the degree of dilution of the hemolymph. The water content in the hemolymph of crabs belonging to the size group 22–26 mm range from 92.7% to 95.05% with a mean of 93.72%. For size group 30–34 mm, the water content ranges from 94.21 to 92.57 with a mean of 92.94%, indicating that the water content does not differ from that of the former group. It is obvious that the observed variations in the THC in different size groups may be due to some factors associated with the age of the individuals. What is interesting in this connection is that even among size groups 30 mm, 33 mm and 34 mm, the THC varied from 3723 cells/mm^3 to 10,062 cells/mm^3. It may be recalled that the reproductive stage of the animal (ovigerous females)in this size range is the same and the blood water content is also constant in these animals.

Time of day as a factor influencing THC

Figure 2 shows that time of day could influence the THC. The THC of forenoon analyses are somewhat lower than afternoon analyses. It may be seen that the value of THC is low during 11:00 to 11:45 AM (5750 ± 1534 cells/mm^3) and increases to 9638 ± 2628 during 3:00 to 3:45 PM. This value subsequently declines steadily.

Effect of thermal stress on THC

Data obtained in the present study also show that thermal stress (either heat or cold) to animals, at a period when counts are minimal, may drive the hemocytes into circulation. THC at 20° C ranges from 6403 cells/mm^3 to 9610 cells/mm^3 with a mean of 7718 ± 1212 cells/mm^3. This value is significantly higher than that of animals analyzed at 30° C. THC at 30° C ranges from 3723 cells/mm^3 to 7648 cells/mm^3, with a mean of 5751 ± 1594 cells/mm^3. Heat treatment at 40° C reduces the count to a mean of 3598 ± 1447 cells/mm^3 with a range of 1936 cells/mm^3 to 5747 cells/mm^3. The average water content of the hemolymph of six animals subjected to thermal stress at 20° C, 30° C, and 40° C is 91.5% and 93.5% and 92.0%, respectively.

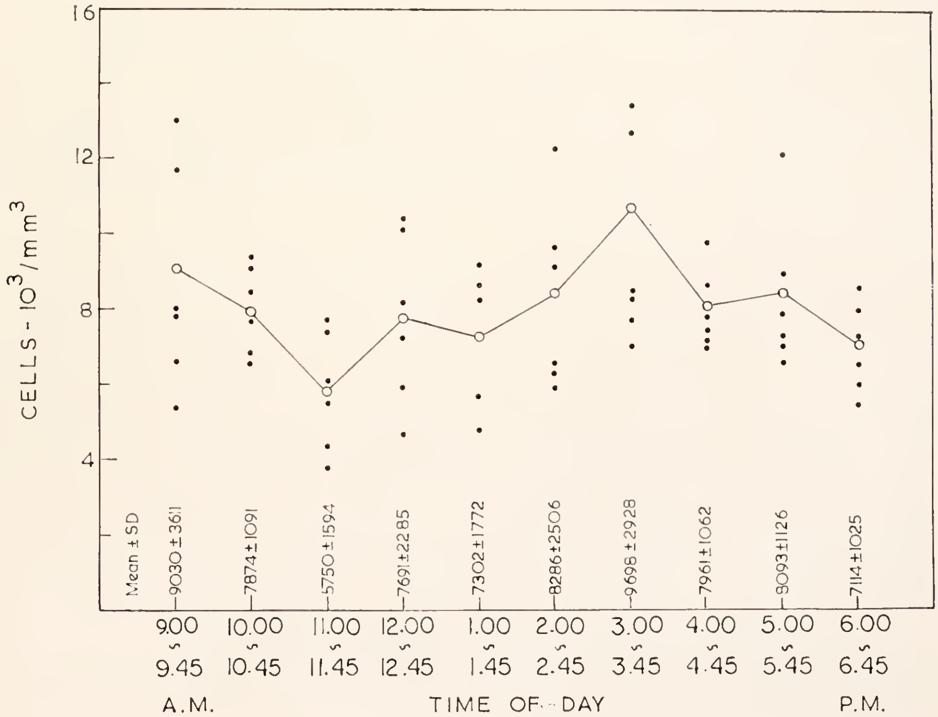

FIGURE 2. Variation in numbers of circulating hemocytes in relation to time of day. The mean values for each size group are shown as circles. The sample size analyzed each hour is six in all cases.

Discussion

The normal count of circulating hemocytes of *Emerita asiatica* was determined after minimizing the inaccuracies caused due to techniques. Errors in counts caused by diluents were avoided. Use of glass pipettes, to which cells adhere (Garvin, 1961), was avoided. The capillary force with which the fresh blood filled the chamber facilitated good distribution and settlement of cells and reduced the magnitude of the field error (see Table I). During the course of this study a new problem arose. As a result of differences between first and subsequent drops used to fill both chambers of the hemocytometer, the question became which one of these values should be used to establish a normal count. Under a similar situation in an insect, Feir (1964) preferred to use the second drop (although it was approximately half the number obtained from the first drop of hemolymph) because it did not show a wide range like that of first drop. In *Emerita asiatica*, both the first and subsequent drops showed a similar range. Therefore an average of both counts was taken.

One of the possible reasons for the wide range in total hemocyte counts of female intermolt *Emerita asiatica*, even after minimizing the technical and observational sources of errors, could be the size of the animals (Fig. 2). Even after controlling the size, the population of hemocytes showed fluctuation in relation to time of day. Although it would be necessary to do sequential sampling over an extended period of time to confirm whether or not there is any daily rhythm in the THC variations, the results suggest possible redistribution of circulating hemocytes during time of day. Such variations in the hemocyte population may occur as a result of the action of any one or a combination of the following mechanisms: alterations in the water content of the blood; alterations in the rate of production of cells; alteration in the rate of destruction of cells; or redistribution of cells in circulation.

The observed variations in THC in different size groups and in relation to time of day may not be due to any change in the concentration of hemolymph, since hemolymph water content did not show significant variation under these conditions. The variations could possibly be due to differences in the rate of production or destruction or hemocytes. In this connection, the observations of Shapiro (1968) pertaining to the dynamics of hematological equilibrium of the larvae of an insect *Galleria mellonella* is of considerable interest. Shapiro (based on the mean values of heat-fixed data, a procedure known to drive hemocytes into circulation) calculated that 76 mitotic cells were found to form a new hemocyte complex approximately every hour, which would contribute to an increase of 3648 hemocytes after a day. Similarly, he found that 921 hemocytes degenerate per hour, which would amount to destruction of 22,104 cells per day. Yet the actual normal of THC is about 37,000 cells per microliter. If the above assumptions of the author were correct, both the rate of production and destruction of cells could not account for the normal hematological equilibrium; moreover, in the absence of an authentic hemocytopoietic tissue in the larvae (see Jones, 1970), it is reasonable to expect some other mechanism contributing to the maintenance of hematological equilibrium.

The fall in hemocyte count could be due to retirement of hemocytes from circulation. Cells thus retired may adhere to tissues or may be sequestered and stored or destroyed. Cells stored by tissues may come out and re-enter circulation. Possibly such a mechanism may operate both in *Emerita asiatica* and also in the insect, *Galleria mellonella*. Observations by Jones (1968, as cited in 1970) that loose accumulations of cells are found near the wing discs of *Galleria mellonella* support the above suggestion. A critical perusal of the review of Jones (1970) would also indicate that the so-called hemocytopoietic tissues reported by a number of investigators in most insects (see Jones, 1970) appear to be such an accumulation of hemocytes resulting from retirement of circulating hemocytes rather than authentic hemocytopoietic organs.

The experiments carried out in this study in order to verify whether the low THC that occurs between 11:00 AM and 11:45 AM is due to retirement of cells from circulation confirm the above suggestion. The results further suggest that environmental conditions may also influence the hematological equilibrium of the animal. It may be noted that in the above conditions the THC does not increase beyond a particular level and falls within the range of THC of the size groups. This feature, together with the fall in THC that occurs in the aging animals (of size groups 30–34 mm), suggests that those hemocytes that retire from circulation in aging animals might be destroyed in tissues to which they adhere, probably after sequestration by tissues, as in vertebrates. Redistribution of circulating hemocytes in tissues reaffirms the earlier suggestion (Ravindranath, 1974) that this mechanism is the basis of many, if not all, of the physiological and pathological variations in the hemocyte population.

The results of this investigation also elucidate some aspects pertaining to the life cycle of hemocytes in *Emerita asiatica*. The life span of hemocytes can now be arbitrarily divided into three phases. First, the phase of hemocytopoiesis, the initiation of development of hemocytes from their stem cell to the times at which they are liberated into circulation; secondly, the circulating phase, in which the hemocyte is in circulation; and thirdly, the noncirculating phase when the hemocyte is out of circulation in the tissue proper. Information available for arthropods is restricted to production of hemocytes. In insects, hemoctyes arise both from hemocytopoietic tissue (Hoffmann, 1970) and also from mitosis of hemocytes in circulation (see Jones, 1970). In several crustaceans [seventeen species of decapods including *Emerita* (= Hippa) *talpodia*], Yeager and Tauber (1935) could not observe more than one mitotic figure in 2000 cells they examined. Although they attributed such low mitotic indices to the presence of leucopoietic tissues in these animals, nothing is known regarding sex, size, nutritional, reproductive or molting conditions of the aniamls investigated. During the present investigation, it was noticed that mitosis is not as rare a feature as reported by the above authors but is restricted to fresh and postmolt animals and possibly to certain times of the day in intermolt ovigerous females. Possibly due to these reasons, mitosis of circulating hemocytes in several arthropods would have been overlooked (see literature cited by Jones, 1970; Ravindranath, 1974).

Furthermore, the present study reveals existence of a noncirculating phase during the life span of hemocytes. Studies made on insects (see Jones, 1970) support the existence of such a phase in the lifespan of hemocytes. This is of interest in all arthropods in view of their open circulatory system.

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SUMMARY

1. The normal count of circulating hemocytes of the mole-crab *Emerita asiatica* was studied by taking into consideration the effects of size, time of the day and thermal stress.

2. Total hemocyte counts were higher in animals belonging to size groups 26–28 mm than in size groups 22–24 mm and 30–34 mm. The water content of hemolymph did not differ among different size groups.

3. Total hemocyte counts also showed variation in relation to time of day. Counts were higher in afternoon than in forenoon. The results suggested a possible redistribution of circulating hemocytes in tissues during various times of the day.

4. The above suggestion was supported by the results of the effect of thermal stress on total hemocytes counts, which revealed that the counts decrease as the temperature increases. The findings are discussed in light of previous studies on hematological equilibrium in invertebrates.

5. The results indicate that the life-span of hemocytes may have three phases: the phase of hemocytopoiesis; the circulating phase; and the noncirculating phase, when the hemocyte is out of circulation.

LITERATURE CITED

BAUCHAU, A. G., AND J. C. PLAQUET, 1973. Variation du numbre des hémocyte chez les crustaces brachyures. *Crustaceana*, **24**: 215–223.

DACIE, J. V., AND S. M. LEWIS, 1968. *Practical hematology*. J. A. Churchill Ltd., London, 568 pp.

DEAN, J. M., AND E. J. VERNBERG, 1966. Hypothermia of blood of crabs. *Comp. Biochem. Physiol.*, **17**: 19–22.

DRACH, P., 1939. Mue et cycle d'intermue chez les crustaces decapodes. *Ann. Inst. Oceanogr.*, **19**: 103–381.

FEIR, D., 1964. Hemocyte counts on the large milk-week bug *Oncopeltus fasciatus*. *Nature*, **202**: 1136–1137.

GARVIN, J. E., 1961. Factors affecting the adhesiveness of human leukocytes and platelets *in vitro*. *J. Exp. Med.*, **114**: 51–73.

HARDY, W. B., 1892. The blood corpuscles of the crustacea, together with a suggestion as to the origin of the crustacean fibrin ferment. *J. Physiol.*, **13**: 165–190.

HOFFMANN, J. A., 1969. Etude de la Récupération hémocytaire après hémorragies expérimentales chez L'orthoptera, *Locusta migratoria*. *J. Insect Physiol.*, **15**: 1375–1384.

HOFFMANN, J. A., 1970. Les organes hemocytopoietiques de deux insects orthropteres: *Locusta migratoria* et *Gryllus binaculatus*. *Z. Zellforsch.* **106**: 451–472.

JONES, J. C., 1970. Hemocytopoiesis in insects. Pages 7–65 in A. S. Gordon, Ed., *Regulation of hematopoiesis, Vol. I.* Appleton Century Crofts, New York.

MATSUMOTO, T., AND M. SAKURAI, 1956. On the volume of the hemocyte in starved silkworm *Bombyx mori* L. *J .Sericult. Sci. Japan*, **25**: 418–422.

RAVINDRANATH, M. H., 1974. Changes in the population of circulating hemocytes during molt cycle phases of the millipede, *Thyropygus poseidon*. *Physiol. Zool.*, **47**: 252–260.

RAVINDRANATH, M. H., 1975a. Effects of temperature on the morphology of hemocytes and coagulation process in the mole-crab *Emerita* (= *Hippa*) *asiatica*. *Biol. Bull.*, **148**: 286–302.

RAVINDRANATH, M. H., 1975b. Effects of hydrogen ion concentration on the morphology of hemocytes of the mole-crab, *Emerita asiatica*. *Biol. Bull.*, **149**: 226–235.

SAWYER, T. K., R. COX, AND M. HIGGINBOTTOM, 1970. Hemocyte value in healthy blue crabs, *Callinectes sapidus* and crabs infected with the amoeba, *Paramoeba perniciosa*. *J. Invert. Pathol.*, **15**: 440–446.

SHAPIRO, M., 1968. Changes in the hemocyte population of the wax moth, *Galleria mellonella*, during wound healing. *J. Insect Physiol.*, **14**: 1725–1733.

STEWART, J. E., J. W. CORNICK, AND J. R. DINGLE, 1967. An electronic method for counting lobster (*Homarus americanus* Milne Edwards) hemocytes and the influence of diet on hemocyte numbers and hemolymph protein. *Can. J. Zool.*, **45**: 291–304.

WIGGLESWORTH, V. B., 1956. The haemocytes and connective tissue formation in an insect, *Rhodnius prolixus* (Hemiptera). *Q. J. Microsc. Sci.*, **97**: 89–98.

YEAGER, J. F., AND O. E. TAUBER, 1935. On the hemolymph cell counts of some marine invertebrates. *Biol. Bull.*, **69**: 66–70.

Reference: *Biol. Bull.*, **152**: 424–436. (June, 1977)

STUDIES ON THE HANDEDNESS OF THE FIDDLER CRAB, *UCA LACTEA*[1]

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The most characteristic feature of the fiddler crab is that the adult male has an enormously developed giant cheliped, while the other remains small; but the female has two small equal-sized chelipeds. The giant cheliped is not present in the early stages of males, but it develops enormously during the period of sexual maturation, and this has been studied by a number of authors. Yerkes (1901) collected numerous male specimens of *Uca pugilator* and *U. pugnax* at random and found that the numbers of right-handed and left-handed individuals were nearly equal. On the basis of this observation, he suggested that the handedness of fiddler crabs is determined primarily by chance. Later, Morgan (1923, 1924), experimenting with some young, sexually immature fiddler crabs, not only concluded that handedness is not determined in the early stages but also claimed that the accidental loss of one cheliped is the sole factor determining handedness. He reported that the giant cheliped always develops on the side of the remaining cheliped. On the other hand, Vernberg and Costlow (1966) claimed after a series of experiments that the handedness is determined genetically. The present author (Yamaguchi, 1973) examined about 5,000 adult males of *U. lactea* and found almost equal numbers of right-handed and left-handed individuals. Gibbs (1974) studied the handedness of male *U. burgersi* and found no significant difference between the numbers of left-handed and right-handed specimens.

In the present paper, observations and experiments designed to elucidate the factors involved in the determination of handedness in male fiddler crabs are presented.

Materials and Methods

Adult specimens of the fiddler crab, *Uca lactea* (de Haan), were collected from 1969 to 1975 from several habitats extending from Amakusa, Kyushu to Ishigaki-Jima, in the Ryukyu Islands (Table I). All the younger crabs and megalopae concerned in this study were collected from 1971 to 1975 from the tidal flats in the vicinity of the Aitsu Marine Biological Station in Amakusa. Megalopae were collected after they settled on the tidal flats, and the young crabs were collected by digging them out of their burrows.

All the megalopae and some of the young crabs were reared in 12 and 20 liter plastic aquariums. Pieces of gravel with a diameter of 3 to 5 cm were placed on the bottom of each aquarium. Over the gravel layer was put a layer of coarse sand

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and over it was placed sandy mud brought from the habitat of the young crabs. A part of the aquarium was left empty and sea water was exchanged through this space. The aquarium was filled with sea water at night, and it was pumped out in the daytime. The crabs were fed powdered dry fish and Wakamoto (a food supplement consisting of vitamins, amino acids, minerals, and proteins) in a ratio of one to three by volume. *U. lactea* is strictly diurnal, and it becomes inactive when it is dark, so each aquarium was illuminated with an incandescent bulb on cloudy days when the laboratory was dark. Two to 36 crabs of approximately the same size were reared together in each aquarium from 1971 to 1975. Most of the crabs were reared until it was clear whether the giant cheliped would be differentiated, usually a period of one to three months. However, some of the crabs were reared until they were sexually mature.

RESULTS

Handedness of adult males

The results of examination of a total of 8088 adult males which were collected from eight local populations are summarized and presented in Table I. Of these 8088, 4071 were right-handed (*i.e.*, the right cheliped was the giant) and the remainder were left-handed. No significant difference from a ratio of 1 : 1 in the

TABLE I

Handedness of adult male U. lactea *having a carapace width greater than 7.0 mm. In Amakusa and Amami-O-Shima, the collection was carried out at three and two different sites, respectively.*

Locality	Year	Right-handed	Left-handed
KYUSHU			
Amakusa, site a	1969	312	315
	1970	902	877
	1971	791	801
	1972	113	117
	1973	482	464
	1974	533	525
	1975	273	273
Amakusa, site b	1970	86	96
Amakusa, site c	1970	51	52
Nichinan	1970	14	17
	1974	26	22
THE RYUKYU ISLANDS			
Amami-O-Shima, site a	1970	69	69
	1973	50	41
Amami-O-Shima, site b	1973	72	71
Okinawa-Jima	1972	30	21
Ishigaki-Jima	1972	169	161
	1973	98	95
Total		4071	4017

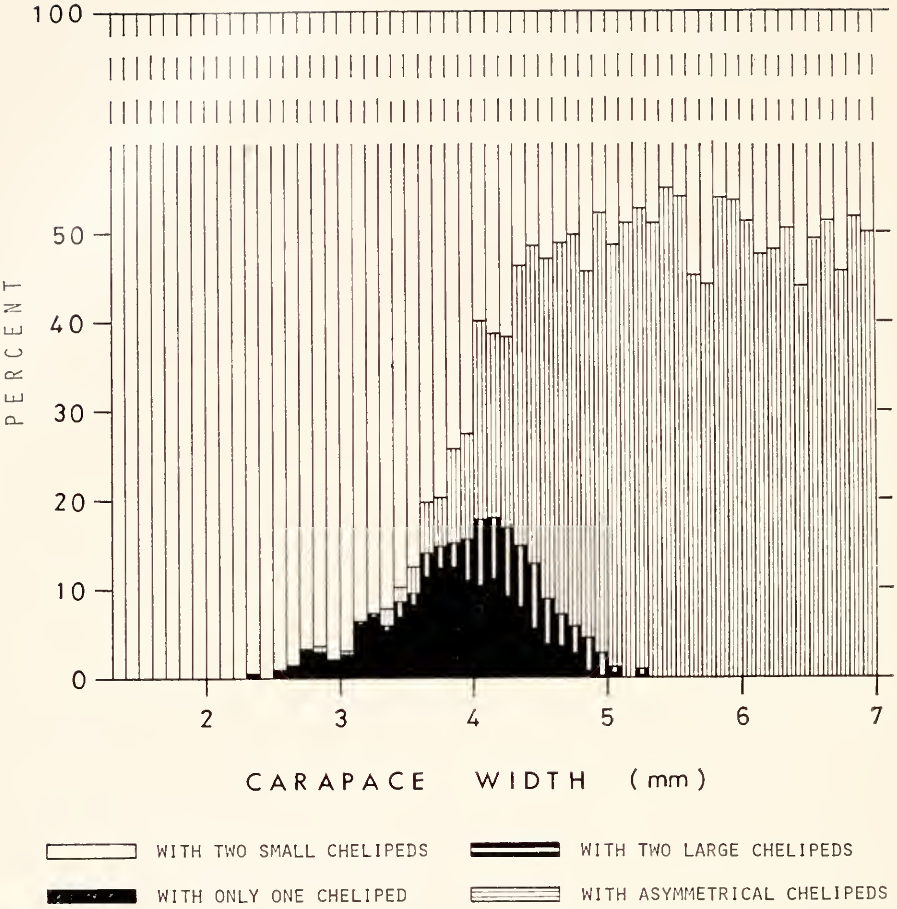

FIGURE 1. Percentage composition of the four kinds of cheliped-types in young crabs of *U. lactea.*

handedness of any of the studied populations was apparent. Furthermore, no significant annual fluctuation in handedness was observed. No adult male having either two giant chelipeds or two small chelipeds was collected.

Chelipeds of young crabs

A total of 13,913 young crabs with a carapace width (CW) less than 7.0 mm was examined. The results are summarized in Figure 1. These data indicate that growth and differentiation of the giant cheliped in males begins in crabs with a carapace width of around 2.5 to 5.0 mm. Almost all the crabs smaller than 2.5 mm still have undifferentiated chelipeds, with both chelipeds being small. Among the transitional crabs, those between the undifferentiated and differentiated stages, those with a carapace width of 2.3 to 5.2 mm, some with only one cheliped and others with two large chelipeds were noticed. Indeed, over 10% of the crabs with a carapace width of around 4 mm had one of these two types of cheliped arrangements. The total number of young crabs lacking their right cheliped was 318, while 293 had lost their left cheliped. The total number of the young crabs with two large chelipeds was 300. The large chelipeds of young males differ in size relative to the carapace width of the giant cheliped from adult males, the small chelipeds of those young males that still have them, and those of adult females. In short, the large chelipeds of the young crabs are intermediate between the small and giant ones.

The numbers of the two sexes were nearly equal after differentiation of the chelipeds had occurred. The sex was determined by examining the abdominal

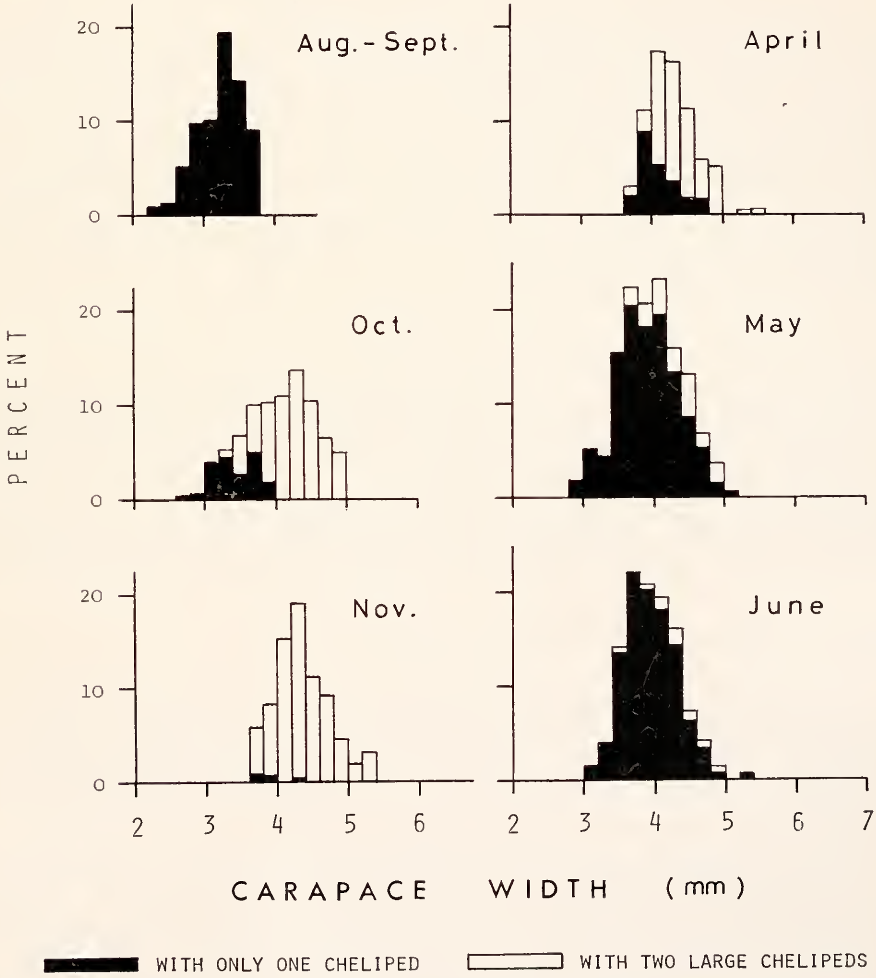

FIGURE 2. Monthly variation of the percentage of young crabs with only one cheliped or with two large chelipeds.

appendage or outer appearance of the abdomen. Both male and female pleopods began to develop at about 3 mm CW, but it was impossible to always distinguish the sexes externally until the crabs became larger than 5 mm CW, for the abdomen of a small-sized female is similar in form and size to that of a young male.

There was a distinct seasonal variation in the percentage of young crabs having only one cheliped or two large chelipeds. In Figure 2, their monthly percentage frequencies are shown. In September and early October young crabs with only one cheliped occurred in high frequency, but those with two large chelipeds were absent or present in only low frequency. The frequency of the former dropped greatly after the middle of October, while that of the latter rapidly increased. In November, of 1307 crabs collected three had only one cheliped, while there were 73 with two large chelipeds.

Young crabs feed and move about actively in September and October, but their activity decreases gradually as the temperature drops in early November, and all the crabs disappear from the surface and hibernate in their burrows during the winter season, from early December to early March. Some of the young crabs begin to appear on the surface about the middle of March, and all have emerged by the end of April. Young crabs with two large chelipeds are still present in high percentage through the middle of April, but the percentage decreases by the end of April. On the other hand, young crabs with only one cheliped increase greatly in number during early May. Most of them had the large cheliped as the remaining one.

Rearing of young crabs with only one cheliped

Eighty-six young crabs having only one cheliped with a 2.3 to 4.4 mm CW (37 were without their right cheliped and 49 without the left one) were brought to the laboratory and reared for 46 days and yielded 53 surviving crabs at the end of rearing. All the survivors were male and attained a CW of 3.8 to 6.2 mm; of these, 26 were left-handed and 27 right-handed. In all cases, the giant cheliped developed on the side that had a cheliped when the crabs were collected, and only a small cheliped formed on the side where one had originally been lost.

Rearing of young crabs with two large chelipeds

In one experiment of this set, 23 crabs with a 4.1 to 5.3 mm CW were reared from the middle of April to the end of May, and all of them survived. They were all males and each discarded one of its chelipeds and the cheliped asymmetry differentiated during this period. Twelve became right-handed males and 11, left-handed ones.

In another experiment, 55 young crabs (3.6 to 4.7 mm CW) were reared from the end of November to July of the following year. Thirty-eight crabs survived. One cheliped was discharded by 35 of these crabs and asymmetry developed. Nineteen males were right-handed and 16 left-handed. The remaining three crabs did not lose a cheliped, and both chelipeds grew equally, each of the three crabs finally developing two giant chelipeds. The sizes of these three males ranged from 9.5 to 10.0 mm CW at the end of rearing. One of them lost its right cheliped as a result of a fight when the crab was about 6 mm CW but regenerated the giant cheliped again and retained the two giant cheliped condition.

The results of rearing show that young crabs with only one cheliped always develop the giant cheliped on the side of the remaining cheliped and the small one forms on the side of lost cheliped. Even in those young crabs that first form two large chelipeds, one of them normally is discarded with the ultimate appearance of cheliped asymmetry in the adult male. Thus, discarding or losing one cheliped seems likely to be the normal process whereby the one-handed character of this species is attained. The three crabs reared in the laboratory that developed and retained two giant chelipeds were exceptional, because no such male was encountered in any of the natural habitats.

Removal of the large cheliped from young males with asymmetrical chelipeds

In order to learn whether cheliped asymmetry could be reversed in this species, the large cheliped was removed from the small-sized males. The young males were easily induced to discard a cheliped by autotomy when it was squeezed by a pair of forceps and pulled lightly. One hundred and fifty-nine males were used; 80 were left-handed and 79 right-handed. They were reared for 58 days after the operation. The body size of the crabs at the beginning of the experiment ranged from 3.2 to 4.1 mm CW, and they grew to 4.8 to 6.8 mm CW by the end of the experiment. One hundred and twenty-six males survived and 124 males retained the same handedness they had before the operation. Two regenerated the small cheliped instead of the giant one and had a female-like appearance.

Another set of experiments was then carried out in which both chelipeds, large and small, were removed simultaneously from young males. One hundred thirteen individuals (50 right-handed and 63 left-handed) were used. Their sizes ranged from 3.4 to 4.5 mm CW. They were kept until the chelipeds regenerated (70–80 days). One hundred and six crabs with a size range of 4.2 to 9.2 mm CW survived. The handedness of the surviving males was exactly the same as before the operation. These results clearly show that the handedness of male crabs is firmly established after the appearance of the asymmetry.

Removal of one cheliped from megalopae and from young crabs that still have two small chelipeds

The right cheliped was removed from 246 megalopae, and they were reared for 73 days, and grew to be crabs of 4.4 to 7.3 mm CW. Forty-four females and 33 males survived until the end of this experiment. All the males developed into left-handed individuals.

The right cheliped was likewise removed from 692 first stage crabs. They were very small in size, 1.3 to 1.5 mm CW, at the time of the operation and were reared for 55 to 65 days by which time they had attained a 5.0 to 6.4 mm CW. The survivors consisted of 193 males and 206 females. The surviving males were all left-handed. The same experiment was performed a second time with 770 slightly larger crabs (1.8 to 4.5 mm CW) that were reared for 55 to 135 days and grew to 3.5 to 7.0 mm CW. Three hundred and eighty-five females and 165 males survived. Of the 165 males, 163 were left-handed, one of the remaining two was right-handed and the second had female-type chelipeds. This female-type male had normally developed male pleopods and his size was 5.8 mm CW. These results are summarized in Table II, according to the sizes of the individuals at the time of operation. There was a large difference between the numbers of surviving males and females when the larger-sized individuals were operated upon, resulting mainly from the difference between the numbers of both sexes at the time of the operation. The percentage of males keeping the two small cheliped condition decreased with the increase in size (Fig. 1), and at approximately 4.5 mm CW, almost all the individuals with two small chelipeds were female.

An experiment in which the left cheliped was removed from 23 young crabs (size 3.3 to 3.5 mm CW) that had two small chelipeds was also performed. Eight females and three right-handed males survived after 246 days.

The results of these experiments indicate that the loss of one cheliped from megalopae and young crabs which still have not exhibited cheliped asymmetry is absolutely essential for the differentiation of handedness in males. The crabs used in this experiment that had two small chelipeds showed no sign of handedness in either sex. Removal of one cheliped resulted in the development of a large cheliped on the other side in almost every instance. This seems to support the assumption that the handedness of this species is not determined in the early stages of growth, but is determined only when a cheliped on one side is lost or removed.

Removal of both chelipeds from megalopae and young crabs with symmetrical chelipeds

Two chelipeds were removed from 35 young crabs, 3.6 to 4.8 mm CW in which two large chelipeds had developed. The crabs were reared for 44 days after the operation and grew to 6.0 to 8.1 mm CW. Twenty-one males survived, but none had a giant cheliped; all chelipeds were small like those of a female.

Both chelipeds were likewise removed from megalopae and young crabs which still had two small chelipeds. A total of 269 megalopae were subjected to the operation and were reared for 296 days, becoming young crabs of 5.6 to 10.3 mm CW. Fourteen females and 15 males survived. Of the males, 13 did not develop a giant cheliped, but possessed only two small chelipeds. It was difficult to rear such young individuals after the operation. Most of them died before metamorphosis. A total of 1098 crabs were also subjected to the operation. Among them, 317 were first stage crabs with a 1.4 to 1.5 mm CW at the time of operation and were reared for 308 to 317 days. Fifty-three females and 47 males with a 4.8 to 8.9 mm CW survived. Forty-two of the 47 males developed only two small chelipeds. An additional 781 crabs had a CW of 2.5 to 4.2 mm, 333 females and 120 males of these having survived until the end of rearing. Of the surviving males, 110 did not develop a giant cheliped. About one-half of the males were reared for 75 days after the operation, but the rearing of other half was continued for 295 to 726 days to observe whether their cheliped condition would change; males with a 6.9 to 13.1 mm CW were finally obtained. No change of the cheliped condition was observed. Most of the males kept two small chelipeds. The results were arranged according to the sizes at the time of the operation and are presented in Table III. One hundred and fifty-two of the

TABLE II

Differentiation of the giant cheliped of U. lactea *after removal of the right cheliped from megalopae and young crabs with two small chelipeds.*

Size at time of operation (CW, mm)	Number of operated crabs	Female	Surviving males		
			Left-handed	Right-handed	With two small chelipeds
Megalopa	246	44	33		
1.3–1.5	692	206	193		
1.8–1.9	103	28	25		
2.2–2.3	42	10	9		
2.4–2.5	59	28	21		
2.6–2.7	65	34	24		
2.8–2.9	32	13	12		
3.0–3.1	34	13	7		
3.2–3.3	24	13	8		
3.4–3.5	31	5	3		
3.6–3.7	94	50	22		
3.8–3.9	92	47	14		
4.0–4.1	100	73	10	1	
4.2–4.3	73	54	7		1
4.4–4.5	21	17	1		
Total	1708	635	389	1	1

167 males obtained in this experiment did not develop the giant cheliped, which seems to be the most characteristic feature of the male fiddler crab, but instead kept two small chelipeds as in female crabs. It was hard to distinguish these males from true females without examining their abdomens. Some of these males which had become sexually mature were bred to mature females in order to determine whether their reproductive system functioned normally. The females laid eggs

TABLE III

Differentiation of the giant cheliped after the removal of two chelipeds from megalopae and young crabs with two small chelipeds.

Size at time of operation (CW, mm)	Number of operated crabs	Female	Surviving males			
			With two small chelipeds	With two large chelipeds	Left-handed	Right-handed
Megalopa	269	14	13	1	1	
1.4–1.5	317	53	42	3	2	
2.5–2.7	216	51	44	3	1	2
2.8–3.0	13	1	1			
3.1–3.3	138	35	23			
3.4–3.6	91	39	8			
3.7–3.9	130	87	14			3
4.0–4.2	193	120	20		1	
Total	1367	400	165	7	5	5

which underwent normal development and hatched. It appears that the males with two small chelipeds obtained from this experiment do have a normal reproductive ability. However, they obviously could not entice females to their burrows by a waving display with the giant cheliped as do normal males (Yamaguchi, 1971). They copulated instead at the entrance of the female burrows. These experiments showed that the simultaneous loss of both chelipeds before the differentiation of asymmetry prevents the development of a giant cheliped.

Removal of one cheliped from the males that regenerated two small chelipeds

To determine the effect of repeated extirpation of one cheliped on the subsequent differentiation of the chelipeds, both chelipeds were first removed from 392 young crabs of a size of 2.7 to 4.3 mm CW. Fifteen to 51 days after the initial operation, all the survivors had regenerated two small chelipeds. The crabs had grown to 2.8 to 7.2 mm CW at the time. Only the right cheliped was then removed again and the crabs were reared for 55 to 70 days following the second operation. One hundred males with a CW of 6.0 to 9.2 mm were obtained. Of these, 94 had two small chelipeds, one was right-handed, three were left-handed, and two had two large chelipeds. Compared to the former experiment, no significant increase of males with a left-handed giant cheliped was obtained. Most of the males with a left-handed giant cheliped was obtained. Most of the males kept two small chelipeds. These results indicate that once the young crabs have suffered simultaneous loss of both chelipeds, the two small cheliped state becomes established, and this condition is not easily changed.

Effect of time interval on removal of two small chelipeds from young crabs

In this series of experiments, both chelipeds were removed from young crabs with two small chelipeds, but instead of the removal being carried out simultaneously, there were various time intervals between the removal of the right cheliped and the subsequent removal of the left cheliped. Seven hundred and forty-seven

TABLE IV

Results of removal of the right cheliped from young crabs, followed by removal of the left one 12 to 192 hours later.

Interval (hr)	Number of operated crabs	Surviving males		
		With two small chelipeds	Left-handed	Right-handed
12	20	6		
24	130	28	5	
48	123	23	10	
72	99	24	10	1
96	241	25	12	2
144	52	4	17	
192	82	1	28	
Total	747	111	82	3

young crabs of the size 2.0 to 3.9 mm CW were used. At the first operation, the right cheliped was removed, and at the second operation, the left cheliped was extirpated. The time intervals between the two operations were 12, 24, 48, 72, 96, 144, and 192 hours. The crabs were examined 150 to 190 days after the second operation, and the resulting data are summarized in Table IV. Of the 747 crabs operated upon, 196 males survived until the end of the experiment. One hundred eleven of these 196 had two small chelipeds, 82 were left-handed, and 3 were right-handed. The left-handed males obtained from these experiments were few in number, about one-third, when the time interval between the first and the second operations was less than 96 hours; but when there was a longer time lapse, the ratio of left-handedness increased dramatically, 17 out of 21 males became left-handed in the 144 hour set and 28 out of 29 in the 192 hour set. It appears that handedness in males of this species is not determined immediately after the artificial loss of both chelipeds from young crabs unless there is a certain length of time between the loss of the two chelipeds. From this experiment, it appears more than 96 hours may be necessary. When the cheliped was removed before this point, a large number of male crabs with two small chelipeds were produced, whereas after this point, most male crabs developed distinct handedness.

DISCUSSION

As has been reported by Morgan (1923, 1924), there are four types of cheliped arrangement in young fiddler crabs, *i.e.,* with two small chelipeds, with two large chelipeds, with one small and one large cheliped, and with one cheliped lost. The appearance of young crabs with two large chelipeds is noted. The results of rearing experiments showed that they are all males and lose one of their chelipeds later in life and thus attain cheliped asymmetry. However, no functional handed-character or morphological difference could be distinguished between the chelipeds before one was lost. A few reared crabs did not lose a cheliped and grew to be adult males with two giant chelipeds. The existence of the stage with two large chelipeds and the appearance of adult males having two giant chelipeds, though no such individual has been collected in its natural habitat, suggest that the chelipeds of young males have an equal potential for growing into the giant in adult males. Based on the results of observations and experiments, the general or fundamental pattern of growth of the asymmetrical chelipeds in male crabs of *Uca lactea* is the following. First, each cheliped has an equal potential to grow and differentiate into a giant cheliped during the early growth period. Secondly, if one cheliped is lost or damaged, the regenerating cheliped loses its potential to differentiate into a giant cheliped. Under natural conditions, the young male loses one of his chelipeds when he reaches a CW of 2.3 to 5.2 mm. The remaining cheliped grows into a giant and cheliped asymmetry is accomplished. Thirdly, if both chelipeds are extirpated simultaneously, both lose the potential, and the crab is unable to ever form a giant cheliped.

The cause of loss of one cheliped at the limited stages of growth is not clear. However, the loss has some relation to the environmental conditions, especially temperature. The frequency of young crabs with only one cheliped was high in October and May (Fig. 2) and the temperature of these months in Amakusa was

19.3° C and 19.0° C, respectively. During the period from November to April, the frequency of young crabs with two large chelipeds increased greatly, and the average temperature during this period was 10.3° C. The seasonal variation of cheliped condition in young crabs can be explained as follows: in the warmer season, young males normally lose one of the chelipeds when they are small in size; while under lower temperatures, the loss of a cheliped may not occur so regularly and growth of the crab is more even, so that some young males grow to the size at which large chelipeds are formed becoming the ones with two large chelipeds. Such males keep their two large cheliped condition temporarily under low temperature, and only lose one of their chelipeds later when it becomes warmer, thus changing into males with only one cheliped. As mentioned above, a few crabs developed and retained two giant chelipeds in the laboratory. However, no such individual was found in 8088 adult males collected in the natural habitat (Table I). It seems certain that the artificial condition in the laboratory prevented the loss of one of the chelipeds; however, the relation between the environmental factors and the process of cheliped loss is not clear at the present. During the warmer seasons the crabs are more active, and the chance of fighting is supposed to increase; however, fighting does not seem to be the usual cause of cheliped loss. A considerable number of young crabs with only one cheliped were found in habitats with a very low population density (below $5/m^2$) and also in ones with very high densities (above $100/m^2$). Young crabs with a CW of 2 to 5 mm feed within a radius of 10 cm from their burrows and rarely stray from this area; therefore, the probability of an encounter and struggle with another individual is very low when the density is lower than $10/m^2$. If fighting is the cause of cheliped loss, there should be some males which have no chance to fight, becoming adult males with two giant chelipeds. No such male was found in any natural habitat. It is highly probable that most young males lose one cheliped spontaneously.

It is clear that the giant chelipeds of adult male fiddler crabs begin to differentiate at a definite period in their life cycle, with a CW of around 2.5 to 5.0 mm. As is well known, the secondary characteristics of many Crustacea are under the control of the androgenic gland (Adiyodi and Adiyodi, 1970; Charniaux-Cotton, 1962; King, 1964). It seems probable that, in the present species also, differentiation of the giant cheliped of males is under the control of the androgenic hormone. Although the development of the androgenic gland in the present species has not been studied, it is highly possible that the cheliped in the male changes in its response as a target organ of the hormone produced from the androgenic gland after its removal or extirpation. If this assumption is reasonable, the competence of the chelipeds for the male hormone might be lost for a short period after the cheliped is damaged or removed.

It has long been known that, when the removal of either one of the chelipeds or both of them is carried out on adult males, no change in handedness occurs when the chelipeds regenerate (Prizibram, 1917). The handedness of adult males, therefore, becomes firmly established once it has appeared. In the present experiments, the removal of the giant cheliped or both of the chelipeds was carried out on younger males, some of which had not experienced molting after the establishment of asymmetry. The result was the same as in the case of adult males, and almost all of the younger males reestablished the same handedness. In this connection, the results of experiments in which the effect of a time interval on removal of two chelipeds offer the interesting notion that the establishment of handedness requires a certain time interval after the loss of one cheliped; in the experiment it was about 96 hours. Ninety-six hours after one cheliped is removed or lost, handedness becomes established firmly, and it cannot be changed by the successive removal of the remaining cheliped. By the successive removal of one cheliped from the males which regenerated two small chelipeds, it became clear that the two small cheliped condition was firmly established in them. The male which developed two giant chelipeds did not change its exceptional cheliped condition after the loss of one of its giant chelipeds. These facts show that once the definitive cheliped condition has been established, further loss or removal of either cheliped is not enough to change the cheliped condition.

The results of the population studies on the handedness of the present species seem to support strongly the conclusion of Yerkes (1901) that the handedness of fiddler crabs is determined by chance. However, as has been reported in a previous paper (Takeda and Yamaguchi, 1973), the natural population of *Uca marionis* showed that almost all of the adult males were right-handed; of the 567 specimens collected, 554 were right-handed and only 13 left-handed. It seems reasonable to consider that the handedness of *U. marionis* is controlled not accidentally but genetically. However, it was possible to induce most of the males to have left-handedness by the removal of the right cheliped when they were in the two small cheliped stage. The mechanism of normal determination of handedness in this species is not clear, but it seems certain that the adult handedness in *U. marionis* also can be predetermined by which one of the small chelipeds is removed in the immature stages (Yamaguchi, unpublished). Vernberg and Costlow (1966) extirpated one cheliped from larger-sized young crabs of *U. rapax* and *U. pugilator* with symmetrical chelipeds. Among the surviving 19 males, five developed a giant cheliped in place of the cheliped removed. Based on this result, they concluded that the handedness of the fidddler crab is determined genetically. It is not clear at present whether the cause of the difference between their results and those of this study depends on the species difference or on a size difference.

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SUMMARY

1. The numbers of right-handed and left-handed males of *Uca lactea* were nearly equal, and no significant regional or yearly differences from the ratio of 1 : 1 were found.

2. In the early stages of growth, all the crabs had two small chelipeds, and no morphological differences were found between the sexes. All males with a carapace width greater than 5.3 mm exhibited cheliped asymmetry. Apparently,

a male normally loses one of his chelipeds which then regenerates into a small cheliped; the remaining cheliped develops into a giant, and the male attains his cheliped asymmetry.

3. Artificial removal of one cheliped from megalopae and young crabs whose chelipeds were still in the symmetrical condition induced handedness. The large cheliped always developed on the intact side.

4. When males suffered the simultaneous removal of both chelipeds in their megalopa or crab stage before the attainment of asymmetry, they could not develop a giant cheliped but instead kept two small chelipeds permanently. However, no abnormality was recognized in their reproductive function. If a male did not lose a cheliped, two giant chelipeds developed.

5. Once the handedness was established in a male, it could not be reversed by the subsequent removal of one or both chelipeds.

LITERATURE CITED

Adiyodi, K. G., and R. G. Adiyodi, 1970. Endocrine control of reproduction in decapod Crustacea. *Biol. Rev.*, **45**: 121–165.

Charniaux-Cotton, H. 1962. Androgenic gland of crustaceans. *Gen. Comp. Endocrinol., Suppl.*, **I**: 241–247.

Gibbs, P. E., 1974. Notes on *Uca burgersi* Holthuis (Decapoda, Ocypodidae) from Bermuda, Leeward Islands. *Crustaceana*, **27**: 84–91.

King, D. S., 1964. Fine structure of the androgenic gland of the crab *Pachygrapsus crassipes*. *Gen. Comp. Endocrinol.*, **4**: 533–544.

Morgan, T. H., 1923. The development of asymmetry in the fiddler crab. *Am. Nat.*, **57**: 269–274.

Morgan, T. H., 1924. The artificial induction of symmetrical claws in male fiddler crabs. *Am. Nat.*, **58**: 289–295.

Prizibram, H., 1917. Transitäre Scherenformen der Winkerkrabbe, *Gelasimus pugnax* Smith. *Arch. Entwick. Mech. Org.*, **43**: 47–62.

Takeda, M., and T. Yamaguchi, 1973. Occurrences of abnormal males in a fiddler crab *Uca marionis* (Desmarest), with notes on asymmetry of chelipeds. *Proc. Jpn. Soc. Syst. Zool.*, **9**: 13–20.

Vernberg, F. J., and J. D. Costlow, Jr., 1966. Handedness in fiddler crabs (genus *Uca*). *Crustaceana*, **11**: 61–64.

Yamaguchi, T., 1971. Courtship behavior of a fiddler crab, *Uca lactea*. *Kumamoto J. Sci. Biol.*, **10**: 13–37.

Yamaguchi, T., 1973. Asymmetry and dimorphism of chelipeds in the fiddler crab, *Uca lactea* De Haan. *Zool. Mag.*, **82**: 154–158.

Yerkes, R. M., 1901. A study of variation in the fiddler crab *Gelasimus pugilator* Latr. *Proc. Am. Acad. Arts Sci.*, **36**: 417–441.

INDEX

A

Acmaea testudinalis, respiration and ecology of, 182
Acontial nematocysts, frequency and mechanism of dart formation in the, of sea anemones, 159
Adaptations, zonational, of intertidal snails, 182
Aerial respiration, intertidal snails, 182
Aiptasia pallida, tentacle and acontial nematocysts of, 159
Allozyme variation, in lobster populations, 41
Altosid, effects on crustacean gametogenesis, 199
Amastigophore, microbasic nematocysts, in sea anemones, 159
Amino acid transport, in *Mytilus*, 295
Amphioplus abditus, larval biology of, 51
Amphipholis squamata, luminescent cells in, 12
Amphiurid, larval biology, 51
Anemone, cerianthid, ptychocyst in, 392
  microbasic q-mastigophore nematocyst in, 159
Anomura, larval development of, 84
Aquatic respiration, intertidal snails, 182
Aragonite crystals, orthorhombic, in Scleractinian corals, 26
Arthropoda, sterility caused by juvenile hormone mimic, 199
  nutrition in, 337
Asterias amurensis, secretory granules of pyloric caeca of, 64
Astrangia danae, intracellular crystals in, 26
Aurelia aurita, ontogeny of swimming behavior in, 233, 251

B

Balanus spp., ovary histology and oogenesis, 351
Barnstable Harbor infaunal communities, 275
Bartholomea annulata, tentacle and acontial nematocysts of, 159
Behavior, clock-compass, in mudsnails, 311
  swimming, in *Aurelia aurita*, 233, 251
Behavioral regulation, of burrow oxygen partial pressure, in *Callianassa*, 134
Benthic community, seasonal changes in structure and species composition of a, 275
Bigger, C. H. See E. J. Conklin, 159; R. N. Mariscal, 392
Bivalve hemolymph lipids, TLC and GLC, 373
Blackwell, J. F., L. F. Gainey, Jr., and M. J. Greenberg. Shell ultrastructure in two subspecies of the ribbed mussel, *Geukensia demissa* (Dillwyn, 1817), 1
Blood cell types, in *Mercenaria*, 105
Boring snails, shell swallowing in, 325
Bradshaw, W. E. and C. M. Holzapfel. Interaction between photoperiod, temperature, and chilling in dormant larvae of the tree-hole mosquito, *Toxorhynchites rutilus* Coq, 147
Brachyuran, limb regeneration, 263
Brain, extirpation and transplantation experiments in tobacco hornworm, 169
Brehm, P. and J. C. Morin. Localization and characterization of luminescent cells in *Ophiopsila californica* and *Amphipholis squamata* (Echinodermata: Ophiuroidea), 12
Brood pouch, structure and function of operculum in *Spirorbis moerchi*, 209
Brown, F. A., Jr. Geographic orientation, time and mudsnail phototaxis, 311
Bunodeopsis antilliensis, tentacle and column vesicle nematocysts of, 159
Busycon, hormonal control of reproduction, 221

C

Calcification, in Scleractinian corals, 26
Callianassa californiensis, use of pleopods in respiration of, 134
Capsule-laying substance, in *Busycon*, 221
Carnivorous mosquitoes, effects of photoperiodism and chilling on, 147
Carriker, M. R. Ultrastructural evidence that gastropods swallow shell rasped during hole boring, 325
Cell types, in blood of *Mercenaria*, 105
Cellular sources, of luminescence, in Ophiuroidea, 12
Cerianthid anemone, ptychocyst in, 392
Chelipeds, closer muscles in *Homarus* larvae, 382
  of young fiddler crabs, 424
  sarcomeres in closer muscles of adult lobster, 75
Childress, J. J. See J. J. Torres, 134
Circulating hemocyte population, in *Emerita asiatica*, 415
Cirripedia, ovary histology and oogenesis, 351
Cladocera nutrition, 337
Classification analysis, of benthic macrofaunal communities, 275

Clibanarius vittatus, larval development of, 84
Clock-compass behavior, in mudsnails, 311
Closer muscle, in adult lobster claw, 75
  in *Homarus* larvae claws, 382
Cluster analysis, of benthic macrofaunal communities, 275
Cnida, new category of, in cerianthid anemone, 392
Cnidaria, microbasic q-mastigophore nematocyst in, 159
  intracellular crystals in, 26
  neuromuscular system, 233
  ontogeny of swimming behavior in, 233, 251
Color polymorphism, in *Crepidula*, 360
Column vesicle nematocysts, frequency and mechanism of dart formation in the, of sea anemones, 159
Community, intertidal benthic, seasonality in, 275
Conklin, D. E. and L. Provasoli. Nutritional requirements of the water flea *Moina macrocopa*, 337
Conklin, E. J., C. H. Bigger, and R. N. Mariscal. The formation and taxonomic status of the microbasic q-mastigophore nematocyst of sea anemones, 159
Conklin, E. J. See R. N. Mariscal, 392
Corals, intracellular crystals in, 12
Costello, W. J. See F. Lang, 75
Costlow, J. D. See U. E. H. Fyhn, 351; G. G. Payen, 199
Crab, fiddler, limb regeneration in, 263
  effect of juvenile hormone mimic on gametogenesis, 199
Crepidula spp., color polymorphism in, 360
Crusher claw, of *Homarus*, muscle fiber types in, 75
Crustacea, circulating hemocyte population, 415
  gametogenesis, effects of juvenile hormone mimic on, 199
  larval development of, 84
Crystals, intracellular, in corals, 26
Culture, laboratory, of hermit crab larvae, 84
Cutter claw, of *Homarus*, muscle fiber types in, 75
Cycloleucine and glycine influx, in *Mytilus*, 295
Cytology, of molluscan hemocytes, 105

D

Dart formation, in sea anemone nematocysts, 159
Development, mosquito, 147
  of embryonic larva, in *Amphioplus abditus*, 51
Diapause maintenance and termination, in mosquito, 147
Differentiation, of chelipeds in *Uca*, 424
Diogenidae, larval development in, 84
Disruptive selection, and color polymorphism, in *Crepidula*, 360
Diurnal variation, of hemocyte count in the mole-crab, 415
Dormancy, mosquito, 147
Drugs and ions, effects on swimming behavior of *Aurelia aurita*, 251

E

Eble, A. F. See C. A. Moore, 105
Ecdysone, association between hemolymph levels of, and radiosensitivity, 169
Echinoderms, larval biology, 51
  bioluminescence in the class Ophiuroidea, 12
Eclosion, radiation-induced inhibition of, in *Manduca sexta*, 169
Ecology, population and community, of infaunal invertebrates, 275
  zonational, of intertidal snails, 182
Egg capsule laying, in *Busycon*, 221
Electron microscopy, of operculum in *Spirorbis moerchi*, 209
  of Scleractinian corals, 26
  of secretory granules of pyloric caeca in starfish, 64
Electrophoresis, enzyme assays of lobster populations, 41
Electrophysiology, of swimming behavior, in *Aurelia aurita*, 233
Ely, M. J., Jr., and A. M. Jungreis. Radiation-induced inhibition of eclosion in the tobacco hornworm, *Manduca sexta*, 169
Embryology, of *Amphioplus*, 51
Emerita asiatica, hemocyte population, 415
Emission spectra, luminescence, of Ophiuroidea, 12
Evolution, rates of organismal and protein, in lobsters, 41

F

Fatty acids, of *Mercenaria* hemolymph, 373
Fertilization membrane, demersal, in *Amphioplus*, 51
Fibers, muscle, in *Homarus* larvae, 382
Fiddler crab, handedness in, 424
  limb regeneration in, 263
Fitness set model, in analysis of polymorphism, in *Crepidula*, 360
Fluorescence, in Ophiuroidea, stimulated to luminesce, 12
Fyhn, U. E. H. and J. D. Costlow. Histology and histochemistry of the ovary and oogenesis in *Balanus amphitrite* L. and *B. eburneus* Gould (Cirripedia, Crustacea), 351

G

GAINEY, L. F., JR. See J. F. Blackwell, 1
Gametogenesis, crustacean, effects of juvenile hormone mimic on, 199
Gas exchange and pleopods, in respiration of *Callianassa*, 134
Gas-liquid chromatography, of *Mercenaria* hemolymph lipids, 373
Gastropoda, intertidal, respiration in, 182
  swallowing of prey shell material, 325
Genetic variation, in *Homarus* species, 41
Geographic orientation and time, and mudsnail phototaxis, 311
Geomagnetism, orientation to, in mudsnails, 311
Geukensia, ultrastructure of shell, 1
Gill surface area, and respiration, in *Callianassa*, 134
GLUCK, D. L. See J. J. Torres, 134
Glycine and cycloleucine influx, in *Mytilus*, 295
GOREAU, N. I. See R. L. Hayes, 26
GOVIND, C. K. See F. Lang, 75, 382
Granules, secretory, of pyloric caeca of *Asterias amurensis*, 64
  types in blood cells of *Mercenaria*, 105
GREENBERG, M. J. See J. F. Blackwell, 1

H

Handedness, of fiddler crabs, 424
HAYES, R. L. AND N. I. GOREAU. Intracellular crystal-bearing vesicles in the epidermis of scleractinian corals, *Astrangia danae* (Agassiz) and *Porites porites* (Pallas), 26
HEDGECOCK, D., K. NELSON, J. SIMONS, AND R. SHLESER. Genic similarity of American and European species of the lobster *Homarus*, 41
Helix aspersa hemolymph, nematode inhibitor from, 406
Hemocytes, in *Mercenaria*, 105
  population in *Emerita asiatica*, 415
Hemolymph, snail, nematode inhibitor from, 406
  cell lipids, of *Mercenaria*, 373
HENDLER, G. Development of *Amphioplus abditus* (Verrill) (Echinodermata: Ophiuroidea): I. Larval biology, 51
Hermit crabs, and shell supplies, 120
  larval development of, 84
Histochemistry, and histology, of *Balanus* ovary, 351
Histology, of photocytes, in Ophiuroidea, 12
HOAGLAND, K. E. A gastropod color polymorphism: one adaptive strategy of phenotypic variation, 360
HOLZAPFEL, C. M. See W. E. Bradshaw, 147
Homarus, genetic similarity in species of, 41
  claw closer muscles in adult, 75
  larvae, claw closer muscles, 382
HORI, S. H., K. TANAHASHI, AND N. MATSUOKA. Morphological and cytochemical studies on the secretory granules of the pyloric caeca of the starfish, *Asterias amurensis*, 64
Hormonal interactions, in arthropod reproductive physiology, 199
Hornworm, tobacco, radiation-induced inhibition of eclosion in, 169
HOSKIN, G. P. AND S. P. HOSKIN. Partial characterization of the hemolymph lipids of *Mercenaria mercenaria* (Mollusca: Bivalvia) by thin-layer chromatography and analyses of serum fatty acids during starvation, 373
HOSKIN, S. P. See G. P. Hoskin, 373
Hybridization, potential for, in *Homarus* species in aquaculture, 41

I

Influx, of amino acids, in *Mytilus*, 295
Inhibition, of eclosion, in *Manduca sexta*, 169
Inhibitor, nematode, from snail hemolymph, 406
Intertidal benthic community, seasonality in, 275
  snails, respiration and ecology, 182
Invertebrates, infaunal, population and community ecology of, 275
Ions and drugs, effects on swimming behavior of *Aurelia aurita*, 251

J

JUNGREIS, A. M. See M. J. Ely, Jr., 169
Juvenile hormone mimic, effects on crustacean gametogenesis, 199

K

Kinetics, of amino acid transport through mussel epidermis, 295

L

Laboratory rearing, of hermit crab larvae, 84
Laboratory culture, of the water flea, 337
Lacuna vincta, respiration and ecology of, 182
LANG, F., W. J. COSTELLO, AND C. K. GOVIND. Development of the dimorphic claw closer muscles of the lobster *Homarus americanus*: I. Regional distribution of muscle fiber types in adults, 75

Lang, F., C. K. Govind, and J. She. Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus:* II. Distribution of muscle fiber types in larval forms, 382
Lang, W. H. and A. M. Young. The larval development of *Clibanarius vittatus* (Bosc) (Crustacea: Decapoda; Diogenidae) reared in the laboratory, 84
Larval biology, of *Amphioplus abditus*, 51
Larval development, of claw closer muscles, in *Homarus*, 382
  of *Clibanarius vittatus*, 84
Light microscopy, of Scleractinian corals, 26
Limb regeneration, in fiddler crabs, 263
Lipid, in bivalve hemolymph, 373
Littoral snails, respiration and ecology of, 182
Lobster, genetic variability in populations of, 41
  larvae closer muscles in, 382
  closer muscles in adult claw, 75
Luminescent cells, in Ophiuroidea, 12

M

Macrobenthos, population and community ecology of, 275
Male fiddler crab, handedness in, 424
Manduca sexta, radiation-induced inhibition of eclosion in, 169
Mariscal, R. N., E. J. Conklin, and C. H. Bigger. The ptychocyst, a major new category of cnida used in tube construction by a cerianthid anemone, 392
Mariscal, R. N. See E. J. Conklin, 159
Matsuoka, N. See S. H. Hori, 64
McMahon, R. F. and W. D. Russell-Hunter. Temperature relations of aerial and aquatic respiration in six littoral snails in relation to their vertical zonation, 182
Medusa, swimming behavior in *Aurelia aurita*, 233, 251
Megalopa stage, of hermit crab larvae, 84
Mercenaria hemocytes, 105
Mercenaria, hemolymph fatty acids during starvation, 373
Mercury, effects on limb regeneration, in fiddler crabs, 263
Methoprene, effect on crab gametogenesis, 199
Methylmercury, effects on limb regeneration, in fiddler crabs, 263
Mitrella lunata, respiration and ecology, 182
Moina macrocopa, nutrition, 337
Mole-crab, hemocyte population, 415
Molluscan hemocytes, cytology of, 105
  hemolymph lipids, 373
Morin, J. G. See P. Brehm, 12
Moore, C. A. and A. F. Eble. Cytochemical aspects of *Mercenaria mercenaria* hemocytes, 105
Morphology, shell, in *Geukensia*, 1
Mosquito, photoperiodism and seasonal development in, 147
Mudsnail, clock-compass behavior, 311
Muricidae, swallowing of prey shell material, 325
Muscle, fast and slow fibers, in *Homarus* larval claws, 382
  fast and slow closer, in adult lobster claw, 75
Mussel, amino acid transport in, 295
  ribbed, shell ultrastructure, 1
Mytilus californianus, amino acid transport in, 295
Mytilus edulis, shell material of, swallowed by preying gastropods during hole boring, 325

N

Nassarius obsoletus, clock-compass behavior, 311
Nelson, K. See D. Hedgecock, 41
Nematocyst, new type in sea anemones, 159
  of cerianthid anemone, 392
Nematode inhibitor, from *Helix aspersa* hemolymph, 406
Nervous system extracts and egg-laying, in *Busycon*, 221
Net flux, of amino acids, in *Mytilus*, 295
Neurohormones, in *Busycon*, 221
Nutrition, of water flea, 337

O

Oogenesis, in *Balanus*, 351
  of crustaceans, effects of juvenile hormone mimic on, 199
Operculum, structure and function in *Spirorbis moerchi*, 209
Ophiopsila californica, luminescent cells in, 12
Ophiuroidea, larval biology, 51
  bioluminescence in, 12
Ovary, histology, in *Balanus*, 351

P

Pagurus spp., population dynamics of, 120
Parietal ganglia, in *Busycon*, egg capsule laying substance in, 221
Payen, G. G. and J. D. Costlow. Effects of a juvenile hormone mimic on male and female gametogenesis of the mud-crab, *Rhithropanopeus harrisii* (Gould) (Brachyura: Xanthidae), 199

Phase contrast microscopy, of sea anemone nematocysts, 159
Pharate-adults, effects of X-irradiation of *Manduca sexta*, 169
Phenotypic plasticity, in *Crepidula*, 360
Photocytes, morphology of, in Ophiuroidea, 12
Photomicroscopy, of ovary in *Balanus*, 351
Photoperiod and chilling, effects on mosquito dormancy and development, 147
Phototaxis, mudsnail, 311
Pleopods, in *Callianassa californiensis* respiration, 134
P-mastigophore, microbasic nematocysts, in sea anemones, 159
Polychaeta, structure and function of operculum in *Spirorbis moerchi*, 209
Polymorphism, color, in *Crepidula*, 360
Population dynamics, hermit crabs, 120
Population genetics, in *Homarus gammarus*, 41
Population and community ecology, of infaunal invertebrates, 275
Porites porites, intracellular crystals in, 26
Potswald, H. E. Further observations on the structure and function of the operculum in *Spirorbis moerchi* (Serpulidae: Spirorbinae), 209
Predation, and color polymorphism, in *Crepidula*, 360
Progeny studies, in *Homarus* species, 41
Protein, snail hemolymph, inhibitor of nematode development, 406
Protein differences, in *Homarus* species, 41
Proteolytic enzymes, in starfish, 64
Provasoli, L. See D. E. Conklin, 337
Ptychocyst, in cerianthid anemone, 392
Pyloric caeca, of *Asterias amurensis*, secretory granules in, 64

Q

Q-mastigophore, microbasic nematocysts, in sea anemones, 159

R

Radiation-induced inhibition of eclosion, in *Manduca sexta*, 169
Radiochemical assay, of coral tissue, 26
Radiosensitivity, of tobacco hornworm, 169
Ram, J. L. Hormonal control of reproduction in *Busycon:* laying of egg capsules caused by nervous system extracts, 221
Ratanarat-Brockelman, C. Isolation of nematode inhibitor from hemolymph of the snail, *Helix aspersa*, 406
Ravindranath, M. H. The circulating hemocyte population of the mole-crab *Emerita (=Hippa) asiatica* Milne Edwards, 415
Regeneration, in fiddler crabs, 263
Reproduction, hormonal control, in *Busycon*, 221
  inhibition of *Rhabditis maupasi*, 406
Respiration, intertidal snails, 182
  of *Callianassa californiensis*, 134
Rhabditis maupasi, inhibition of development and reproduction by snail hemolymph protein, 406
Rhithropanopeus harrisii, effect of juvenile hormone mimic on gametogenesis, 199
Russell-Hunter, W. D. See R. F. McMahon, 182

S

Salinity tolerance, in embryo of *Amphioplus*, 51
Sarcomere fibers, in adult lobster claw closer muscles, 75
  in claw closer muscles of *Homarus* larvae, 382
Scanning electron microscopy, of cerianthid anemone, 392
  of sea anemone nematocysts, 159
  of shell material rasped during hole boring, 325
  of shells in *Geukensia*, 1
Schyphistoma, tentacular system, of *Aurelia aurita*, electrophysiology and responses to ions and drugs, 233, 251
Scleractinian corals, intracellular crystals in, 26
Schwab, W. E. The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita*. I. Electrophysiological analysis, 233
  The ontogeny of swimming behavior in the scyphozoan, *Aurelia aurita*. II. The effects of ions and drugs, 251
Sea anemone, microbasic q-mastigophore nematocysts in, 159
  cerianthid, ptychocyst in, 392
Searlesia dira shells, use of by hermit crabs, 120
Seasonality, in an intertidal benthic community, 275
Secretory granules, of pyloric caeca of *Asterias amurensis*, 64
Sedimentary parameters, influence on trophic and species composition of a macrobenthic community, 275
Serpulidae, structure and function of operculum in *Spirorbis moerchi*, 209
She, J. See F. Lang, 382

Shell, availability and use, by hermit crabs, 120
  prisms, and lamellae, from *Mytilus edulis,* in stomach of *Urosalpinx cinerea,* 325
  swallowing, in boring snails, 325
  ultrastructure, in *Geukensia,* 1
Shileser, R. See D. Hedgecock, 41
Shrimp respiration, 134
Simons, J. See D. Hedgecock, 41
Size effects, on count of circulating hemocytes of the mole-crab, 415
Snails, intertidal, respiration in, 182
  hemolymph, nematode inhibitor from, 406
  shells, use by hermit crabs, 120
Species differences and regeneration, in fiddler crabs, 263
Spermatogenesis, of crustaceans, effect of juvenile hormone mimic on, 199
Spight, T. M. Availability and use of shells by intertidal hermit crabs, 120
Spirorbis moerchi, structure and function of operculum in, 209
Starfish, secretory granules of pyloric caeca of, 64
Strobilation, in *Aurelia aurita,* 233, 251
Sublittoral snails, respiration and ecology, 182
Swimming behavior, in *Aurelia aurita,* 233, 251

T

Tanahashi, K. See S. H. Hori, 64
Temperature, effects on mosquito dormancy and development, 147
  compensation of mosquito photoperiodic clock, 147
  relations in snail respiration, 182
  tolerance in embryo of *Amphioplus,* 51
Tentacle nematocysts, frequency and mechanism of dart formation in the, of sea anemones, 159
Thais sp., shells, use of by hermit crabs, 120
Thermal stress, effects on count of circulating hemocytes of the mole-crab, 415
Thin layer chromatography, of *Mercenaria* hemolymph lipids, 373
Time and geographic orientation, in mudsnail phototaxis, 311
Tobacco hornworm, radiation-induced inhibition of eclosion in, 169
Torres, J. J., D. L. Gluck, and J. J. Childress. Activity and physiological significance of the pleopods in the respiration of *Callianassa californiensis* (Dana) (Crustacea: Thalassinidea), 134
Toxorhynchites rutilus, photoperiodism and seasonal development, 147
Transmission electron microscopy, of cerianthid anemone, 392
Transport, amino acid, in *Mytilus,* 295
Tube construction, by cerianthid anemone, 392

U

Uca spp., limb regeneration in, 263
Uca lactea, handedness, 424
Ultrastructure, of cerianthid anemone, 392
  of operculum in *Spirorbis moerchi,* 209
  of Scleractinian corals, 26
  of sea anemone nematocysts, 159
  of secretory granules of pyloric caeca in starfish, 64
  of shell, in *Geukensia,* 1
  of shell material rasped from bore hole by gastropods during penetration of prey, 325
Urosalpinx cinerea follyensis, swallowing of prey shell material, 325

V

Vertical distribution, of snails in the intertidal environment, 182

W

Water flea, nutrition, 337
Weis, J. S. Limb regeneration in fiddler crabs: species differences and effects of methylmercury, 263
Whitlatch, R. B. Seasonal changes in the community structure of the macrobenthos inhabiting the intertidal sand and mud flats of Barnstable Harbor, Massachusetts, 275
Wright, S. H. and G. C. Stephens. Characteristics of influx and net flux of amino acids in *Mytilus californianus,* 295

Y

Yamaguchi, T. Studies on the handedness of the fiddler crab, *Uca lactea,* 424
Young, A. M. See W. H. Lang, 84

Z

Zoeal stages, of hermit crab larvae, 84
Zonation, vertical, of intertidal snails, 182
Zymogen form, of proteolytic enzymes, in starfish, 64

Continued from Cover Two

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CONTENTS

BROWN, F. A., JR.
Geographic orientation, time and mudsnail phototaxis........... 311

CARRIKER, MELBOURNE R.
Ultrastructural evidence that gastropods swallow shell rasped during hole boring.. 325

CONKLIN, DOUGLAS E. AND LUIGI PROVASOLI
Nutritional requirements of the water flea *Moina macrocopa*...... 337

FYHN, UNNI E. H. AND JOHN D. COSTLOW
Histology and histochemistry of the ovary and oogenesis in *Balanus amphitrite* L. and *B. eburneus* Gould (Cirripedia, Crustacea)...... 351

HOAGLAND, K. ELAINE
A gastropod color polymorphism: one adaptive strategy of phenotypic variation.. 360

HOSKIN, GEORGE P. AND SOMSONG P. HOSKIN
Partial characterization of the hemolymph lipids of *Mercenaria mercenaria* (Mollusca: Bivalvia) by thin-layer chromatography and analyses of serum fatty acids during starvation............. 373

LANG, FRED, C. K. GOVIND AND JOSEPH SHE
Development of the dimorphic claw closer muscles of the lobster, *Homarus americanus:* II. Distribution of muscle fiber types in larval forms.. 382

MARISCAL, RICHARD N., EDWIN J. CONKLIN AND CHARLES H. BIGGER
The ptychocyst, a major new category of cnida used in tube construction by a cerianthid anemone.......................... 392

RATANARAT-BROCKELMAN, CHARIYA
Isolation of nematode inhibitor from hemolymph of the snail, *Helix aspersa*.. 406

RAVINDRANATH, M. H.
The circulating hemocyte population of the mole-crab *Emerita* (=*Hippa*) *asiatica* Milne Edwards........................... 415

YAMAGUCHI, TAKAO
Studies on the handedness of the fiddler crab, *Uca lactea*........ 424

INDEX TO VOLUME 152....................................... 437

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